UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA, TOXICOLOGÍA Y MEDICINA LEGAL

"DESARROLLO Y EVALUACIÓN TOXICOLÓGICA DE NUEVOS MATERIALES PARA SU APLICACIÓN EN LA CONSERVACIÓN DE ALIMENTOS"

Memoria que presenta la Licenciada SARA MAISANABA HERNÁNDEZ para optar al título de Doctor por la Universidad de Sevilla con la Mención Internacional

Sevilla, 2015

UNIVERSIDAD DE SEVILLA

AREA DE TOXICOLOGÍA
C/ Profesor García González, nº 2
41012 Sevilla (España)
Teléfono: 954 55 67 62
Fax: 954 55 64 22

Dª ANA MARÍA CAMEÁN FERNÁNDEZ, Catedrática de Universidad y Directora del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla,

CERTIFICA: Que la Tesis Doctoral titulada "**DESARROLLO Y EVALUACIÓN TOXICOLOGÍCA DE NUEVOS MATERIALES PARA SU APLICACIÓN EN LA CONSERVACIÓN DE ALIMENTOS**", presentada por la Lda. Dª SARA MAISANABA HERNÁNDEZ para optar al grado de Doctor por la Universidad de Sevilla con la Mención Internacional, ha sido realizada en el Área de Toxicología de este Departamento bajo la dirección de la Dra. Ángeles Mencía Jos Gallego y Dra. Silvia Pichardo Sánchez. Así mismo, se incluyen las investigaciones llevadas a cabo en el Instituto Nacional de Biología (National Institute of Biology, NIB) de Ljubljana, Eslovenia, bajo la dirección de la Dra. Metka Filipic.

Y para que así conste, firmo el presente en Sevilla,a 9 de Septiembre de 2015.

FACULTAD DE FARMACIA
Departamento Nutrición y Bromatología,
Toxicología y Medicina Legal

Dra. Ana María Cameán Fernández

UNIVERSIDAD DE SEVILLA

AREA DE TOXICOLOGÍA
C/ Profesor García González, nº 2
41012 Sevilla (España)
Teléfono: 954 55 67 62
Fax: 954 55 64 22

Dª ÁNGELES MENCÍA JOS GALLEGO Y Dª SILVIA PICHARDO SÁNCHEZ, Profesoras Titulares del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla,

INFORMAN: Que siendo directoras de la Tesis Doctoral de la Licenciada Dª SARA MAISANABA HERNÁNDEZ, con título "**DESARROLLO Y EVALUACIÓN TOXICOLOGÍCA DE NUEVOS MATERIALES PARA SU APLICACIÓN EN LA CONSERVACIÓN DE ALIMENTOS**", AVALAN la idoneidad de la presentación de la misma en la modalidad de COMPENDIO POR PUBLICACIONES.

Y para que así conste, firmamos el presente en Sevilla, a 9 de Septiembre de 2015.

FACULTAD DE FARMACIA
Departamento Nutrición y Bromatología, Toxicología y Medicina Legal

Dra. Ángeles Mencía Jos Gallego

Dra. Silvia Pichardo Sánchez

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41012 Sevilla (España)
Teléfono: 954 55 67 62
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CERTIFICAN: Que la Tesis Doctoral titulada "**DESARROLLO Y EVALUACIÓN TOXICOLOGÍCA DE NUEVOS MATERIALES PARA SU APLICACIÓN EN LA CONSERVACIÓN DE ALIMENTOS**", ha sido realizada por la Lda. Dª SARA MAISANABA HERNÁNDEZ en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar a la mención de Doctor Internacional por la Universidad de Sevilla.

Y para que así conste, firmamos el presente en Sevilla, a 9 de Septiembre de 2015.

Dra. Ángeles Mencía Jos Gallego

FACULTAD DE FARMACIA
Departamento Nutrición y Bromatología,
Toxicología y Medicina Legal

Dra. Silvia Pichardo Sánchez

UNIVERSIDAD DE SEVILLA

AREA DE TOXICOLOGÍA
C/ Profesor García González, nº 2
41012 Sevilla (España)
Teléfono: 954 55 67 62
Fax: 954 55 64 22

Este trabajo ha sido realizado en el Área de Toxicología del Departamento de Nutrición y Bromatología, Toxicología y Medicinal Legal de la Facultad de Farmacia de la Universidad de Sevilla, y se ha financiado con los siguientes Proyectos de Investigación:

- P10-AGR-5969, Proyecto de Excelencia de la Junta de Andalucía: Desarrollo y Evaluación Toxicológica de Nanomateriales para su Aplicación en Conservación de Alimentos (Investigadora Principal: Ángeles Mencía Jos Gallego).
- AGL2010-21210, Plan Nacional de 2010 (Ministerio de Economía y Competitividad): Desarrollo y Evaluación Toxicológica de Nanoarcillas de Aplicación en el Envasado de Alimentos (Investigadora Principal: Ángeles Mencía Jos Gallego).
- AGL2012-38357-C02-01, Plan Nacional de 2012 (Ministerio de Economía y Competitividad): Evaluación de la Seguridad para Contacto Alimentario de Envases Activos Basados en Polímeros Biodegradables y Extractos Naturales (Investigadora Principal: Ana María Cameán Fernández).
- P11-AGR-7252, Proyecto de Excelencia de la Junta de Andalucía: Desarrollo y Evaluación Toxicológica de Envases Activos Aptos para Contacto Alimentario (Investigadora Principal: Ana María Cameán Fernández).

La Doctoranda Dª Sara Maisanaba Hernández ha disfrutado de una ayuda Predoctoral de Personal Investigador en Formación (PIF), asociada a un Proyecto de Excelencia de la Junta de Andalucía (P10-AGR- 5969), desde Febrero de 2012.

Así mismo, para la realización de la estancia en el extranjero, la Doctoranda Dª Sara Maisanaba Hernández ha disfrutado de una Ayuda de Movilidad derivada del V Plan Propio de Investigación de la Universidad de Sevilla (2014).

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ADN: ácido desoxirribonucleico

Al: aluminio

AlO_6: óxido de alumnio

APTES: aminopropiltrietoxisilano

ARN: ácido ribonucleico

ARN: ácido ribonucleico mensajero

Ca: calcio

Caco-2: línea celular humana de adenocarcinoma de colon

CAT: catalasa

CEC: capacidad de intercambio iónico

CHO: células de ovario de hámster chino

CITIUS: Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla

CNa^+: Cloisite®Na^+

C15A: Cloisite®15A

C10A: Cloisite®10A

C20A: Cloisite®20ª

C93A : Cloisite®93A

d: espesor de la membrana polimérica

***d´*:** trayectoria/longitud de la difusión del gas en presencia de las nanoláminas de arcillas

EE.UU: Estados Unidos

EFSA: European Food Safety Authority

ERO: especies reactivas de oxígeno

FDA: Food and Drug Administration

Fe: hierro

FTIR: espectroscopía infrarroja

g: gramo

GPX: glutatión peroxidasa

GR: glutatión reductasa

GRAS: Generally Recognized as Safe

GSH: glutatión

h: horas

HEK293: línea celular renal

HeLa: línea celular epitelial

HepG2: línea celular humana de hepatoma

HMy2.CIR: línea celular linfoblástica

HUVEC: línea celular humana de vena endotelial umbilical

IA: industria alimentaria

IARC: Agencia Internacional de Investigación sobre el Cáncer

IL-6: interleucina-6

IMR32: línea celular neuroblástica

IMR90: línea celular fibroblástica

INT-407: línea celular intestinal

kg: kilogramo

L: espesor de las nanoláminas de arcilla

L5178Y: línea celular linfoide

L929: línea celular fibroblástica

LDH: lactato deshidrogenasa

Li: litio

LPO: peroxidación lipídica

M: molar

MCF-7: línea celular epitelial

MDA: malondialdehído

mequiv.: miliequivalente

mg: miligramo

mL: mililitro

Mg: magnesio

MgO_6: óxido de magnesio

MLA: mouse lymphoma assay/ ensayo de linfoma de ratón

mM: milimolar

MN: micronúcleos

Mt: montmorillonita

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium

MTT: Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol

N1E-115: línea celular neuronal

N2a: línea celular neuronal

Na: sodio

NIB: National Institute of Biology

NIH3T3: línea celular fibroblástica

nm: nanómetro

O^2: oxígeno

OCDE: Organización para la Cooperación y Desarrollo Económico

OMS: Organización Mundial de la Salud

PCR: reacción en cadena de la polimerasa

PET: polietileno

PLA: ácido poliláctico

PP: polipropileno

ppm: partes por millón

q-PCR: PCR cuantitativa a tiempo real

RN: rojo neutro

ROC: línea celular neuronal

S9: fracción metabólica externa

SCE: intercambio de cromátidas hermanas

Si: silicio

SiO4: tetaedro de silicato

SOD: superóxido dismutasa

TGA: termogravimetría

TNF-α: factor de necrosis tumoral-α

U937: línea celular monocítica

UE: Unión Europea

V79: línea celular pulmonar

VMTS: viniltrimetoxisilano

W: grosor de las nanoláminas de arcilla

µg: microgramo(s)

µmol: micromol(es)

µm: micrómetro

µM: micromolar

I. RESUMEN / SUMMARY

RESUMEN

En la actualidad, la industria alimentaria está apostando por la incorporación de sustancias naturales a envases alimentarios con el fin de incrementar la perdurabilidad del alimento en el mercado. Debido al empleo de estas sustancias en esta nueva aplicación, la seguridad de los consumidores y del medio ambiente puede verse comprometida debido a una mayor exposición, desconociéndose en gran medida, hasta el momento, las posibles consecuencias. De entre las sustancias naturales seleccionadas por la industria alimentaria para este fin, destacan las arcillas y minerales de arcillas, y los aceites esenciales y sus respectivos componentes mayoritarios, constituyendo las primeras (arcillas) el principal objeto de estudio de la presente Tesis Doctoral. Por todo ello, decidimos realizar una evaluación toxicológica mediante una batería de ensayos tanto *in vitro* como *in vivo*, para investigar los posibles efectos tóxicos que pueden desencadenarse tras la exposición a estas sustancias, tanto puras como ya incorporadas en el envase. La relevancia de la información toxicológica de estas sustancias es tal que constituye un requisito reglamentario por parte de las autoridades competentes antes de su comercialización.

En el caso de las arcillas y minerales de arcilla, se ha demostrado una mejora de las propiedades mecánicas, térmicas y barrera de las matrices poliméricas debido a su incorporación, lo que está, íntimamente relacionado con el incremento de la vida útil de los alimentos. Con el fin de contextualizar la situación ante la que se encontraban las arcillas y sus derivados en el área del envasado de la industria alimentaria y así abordar posteriores estudios, nos pareció importante realizar una exhaustiva revisión bibliográfica sobre los datos de toxicidad publicados en la literatura científica hasta la actualidad. La disparidad de los resultados disponibles hasta el momento hace necesaria una evaluación toxicológica caso por caso. Diferentes parámetros pueden estar involucrados en la respuesta obtenida, incluyendo: (i) condiciones de exposición tales como concentraciones seleccionadas o tiempos ensayados; (ii) modelos experimentales elegidos; (iii) modificadores o surfactantes incorporados a la estructura de la arcilla, (iv) sensibilidad de los ensayos llevados a cabo, etc. Dicha revisión bibliográfica ha dado lugar a la siguiente publicación:

- ***TOXICOLOGICAL EVALUATION OF CLAY MINERALS AND DERIVED NANOCOMPOSITES: A REVIEW.*** *(Maisanaba y col., 2015; Environmental Research 138, 233-254).*

La vía de exposición más importante en el contexto que nos engloba es la vía oral, dado que las arcillas van a ser incorporadas a un envase que posteriormente se va a poner en contacto con un alimento, y éste último será ingerido por los consumidores. En este caso los órganos que principalmente se podrían ver afectados serán los que componen el sistema digestivo, destacando entre ellos el intestino, encargado de la absorción de nutrientes, y el hígado, crucial en la biotransformación de xenobióticos. Se llevó a cabo una batería de ensayos de los materiales objeto de estudio con el fin de esclarecer sus principales efectos tóxicos, teniendo en cuenta la escasez y disparidad de los datos disponibles. Las líneas celulares seleccionadas fueron una línea celular de hepatoma humano (HepG2) y una línea celular de adenocarcinoma de colon (Caco-2). Éstas fueron expuestas a diversas arcillas no modificadas y modificadas con sales de amonio cuaternario, tanto comercializadas (Cloisite®Na^+ (CNa^+), Cloisite®20A (C20A) y Cloisite®30B (C30B)) o desarrolladas por el Instituto Tecnológico de Embalaje, Transporte, y Logística (ITENE) de Valencia (Clay1 y Clay2). Los experimentos llevados a cabo incluyeron la evaluación de: citotoxicidad basal, análisis de la morfología celular, genotoxicidad (ensayo cometa y ensayo de micronúcleos (MN)) y estrés oxidativo (producción de especies reactivas de oxígeno (ERO) y contenido de glutatión (GSH)). De forma general se obtuvo ausencia de toxicidad en el caso de las arcillas CNa^+, C20A y Clay1, y sólo en el caso de la primera arcilla mencionada se obtuvo una inducción de MN significativa y alguna alteración de la morfología celular. Sin embargo, pudimos observar daño en ambas líneas celulares tras la exposición a C30B y Clay2 en la mayoría de los parámetros evaluados. Además, se ha llevado a cabo por primera vez en la línea celular HepG2 el estudio de la influencia de las arcillas en la modulación de la expresión génica de un extenso grupo de genes involucrados en activación/destoxicación y otros mecanismos de acción tóxica. CNa^+ y Clay2 sí demostraron alterar la regulación de distintos genes, no así C30B y Clay1. Los resultados de estos experimentos han dado lugar a las siguientes publicaciones:

- ***IN VITRO TOXICOLOGICAL ASSESSMENT OF CLAYS FOR THEIR USE IN FOOD PACKAGING APPLICATIONS.*** *(Maisanaba y col., 2013, Food and Chemical Toxicology 37, 266-275).*
- ***GENETIC POTENTIAL OF MONTMORILLONITE CLAY MINERAL AND ALTERATION IN THE EXPRESSION OF GENES INVOLVED IN TOXICITY MECHANISMS IN THE HUMAN HEPATOMA CELL LINE HEPG2.*** *(Maisanaba y col., 2015; Journal of Hazardous Materials (en revision/ under revision)).*

- ***INDUCTION OF MICRONUCLEI AND ALTERATION OF GENE EXPRESSION BY AN ORGANOMODIFIED CLAY IN HEPG2 CELLS. (****Maisanaba y col., 2015; Archives of Toxicology (en revisión/ under revision)).*
- ***EFFECTS OF TWO ORGANOMODIFIED CLAYS INTENDED TO FOOD CONTACT MATERIALS ON THE GENOMIC INSTABILITY AND GENE EXPRESSION OF HEPATOMA CELLS.*** *(Maisanaba y col., 2015); Toxicology Letters (en revision/ under revision).*
- ***TOXIC EFFECTS OF A MODIFIED MONTMORILLONITE CLAY ON THE HUMAN INTESTINAL CELL LINE CACO-2.*** *(Maisanaba y col., 2014; Journal of Applied Toxicology 34, 714-725).*
- ***TOXICITY ASSESSMENT OF ORGANOMODIFIED CLAYS USED IN FOOD CONTACT MATERIALS ON HUMAN TARGET CELL LINES.*** *(Houtman y col., 2014; Applied Clay Science 90, 150-158).*

Aunque los órganos y tejidos del sistema digestivo serían probablemente los más afectados por exposición a las arcillas, hay otras dianas que también podrían verse dañadas, destacando el tejido endotelial vascular, involucrado en la distribución y absorción. Hasta el momento los datos disponibles acerca de los efectos de este modelo experimental expuesto a arcillas son limitados. Por ello, se ha llevado a cabo la evaluación de la viabilidad de la línea celular endotelial de vena umbilical humana (HUVEC) expuestas a CNa^{+}, C30B, Clay1 y Clay2. Los resultados obtenidos indicaron que las células HUVEC seguían un patrón de respuesta muy similar al de HepG2 y Caco-2 ante la exposición a las arcillas, presentando una sensibilidad parecida a la línea celular hepática. Además, el estudio del potencial mutagénico mediante el Test de Ames es una prueba exigida antes de la comercialización de cualquier producto que vaya a entrar en contacto con alimentos (EFSA 2011b, 2015), siendo en este caso necesaria la evaluación de las arcillas que estén destinadas a ser incorporadas al envasado alimentario. El modelo experimental seleccionado fue *Salmonella typhimurium* (5 cepas), el cual se expuso a las cuatro arcillas mencionadas, en ausencia y presencia de fracción metabólica externa. Únicamente pudimos observar una respuesta mutagénica positiva en el caso de C30B y Clay1. Los resultados obtenidos se reflejan en la siguiente publicación:

- ***CYTOTOXICITY AND MUTAGENICITY ASSESSMENT OF ORGANOMODIFIED CLAYS POTENTIALLY USED IN FOOD PACKAGING.*** *(Maisanaba y col., 2015; Toxicology In Vitro 29, 1222-1230).*

La Autoridad Europea de Seguridad Alimentaria (EFSA) no sólo propone la evaluación de los materiales que se incorporan a las matrices poliméricas de envasado, sino también, siendo incluso de mayor importancia, la evaluación de los envases resultantes (EFSA, 2011a,b). En este sentido, ITENE desarrolló dos materiales nanocompuestos basados en ácido poli(láctico) (PLA) y Clay1/Clay2, ambas arcillas diseñadas por ellos. Con estos materiales se llevaron a cabo ensayos de migración, utilizando como simulante alimentario agua destilada, aceptado por el Reglamento UE Nº 10/2011 dado el tipo de alimentos hacia los que van dirigidos los mismos (hidrófilos).

Una vez obtenidos los extractos de migración de PLA-Clay1 y PLA-Clay2, se evaluaron distintos parámetros con el fin de determinar los posibles efectos tóxicos debido a la migración de las arcillas tras la exposición a los mismos. En este sentido, se evaluó la viabilidad celular de HepG2 y Caco-2 expuestas a ambos extractos, así como el potencial mutagénico de los mismos mediante el Test de Ames. En los ensayos llevados a cabo se observó la ausencia de citotoxicidad y mutagenicidad en las condiciones ensayadas. Por otro lado, teniendo en cuenta que la presencia de los metales mayoritarios de la estructura de las arcillas son Al, Ca, Fe, Mg y Si, se evaluó el contenido de los mismos en los extractos de migración mediante Espectrometría de Masas con Plasma Acoplado Inductivamente (ICP-MS) y Espectroscopía de Plasma Inductivo acoplado a Espectroscopía de Emisión Óptica (ICP-OES), no obteniéndose diferencias significativas con respecto al grupo control. Estos resultados se recogen en la siguiente publicación:

- ***CYTOTOXICITY AND MUTAGENICITY STUDIES ON MIGRATION EXTRACTS FROM NANOCOMPOSITES WITH POTENTIAL USE IN FOOD PACKAGING.*** *(Maisanaba y col., 2014 Food and Chemical Toxicology 66, 366-372).*

Una vez realizado el estudio toxicológico *in vitro* de las arcillas y considerando las mejoras tecnológicas presentadas por los polímeros nanocompuestos resultantes, se seleccionó la arcilla de mejor perfil toxicológico y tecnológico, siendo en este caso Clay1 la seleccionada para continuar la investigación. Con el fin de completar los resultados obtenidos *in vitro* y dada la escasez de información *in vivo*, se realizó un ensayo de toxicidad subcrónica durante 90 días en ratas Wistar expuestas a Clay1 (40 mg/kg/día) en la dieta y su extracto de migración (PLA-Clay1) como agua de bebida (*ad libitum*). En este sentido, transcurrido el periodo de exposición, los animales fueron sacrificados y se extrajeron sus órganos (hígado,

riñón, intestino, cerebro, corazón, testículos, pulmones y bazo) y sangre mediante punción cardiaca. Se evaluaron una serie de parámetros, incluyendo: análisis histopatológico de todos los tejidos, bioquímica clínica del suero sanguíneo, liberación de interleucina 6 (IL-6), biomarcadores involucrados en la respuesta del posible estrés oxidativo generado en hígado y riñón, tales como la ratio glutatión reducido/glutatión oxidado (GSH/GSSG), peroxidación lipídica (LPO), y actividades de enzimas antioxidantes (superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) y glutatión–S-transferasa (GST). Además, la expresión génica y abundancia proteica de SOD y CAT en hígado y riñón también fueron evaluadas. Podemos destacar un aumento de la actividad de CAT en riñón, así como de expresión génica y abundancia proteica, observado tras la exposición a Clay1. El resto de los marcadores evaluados tras la exposición a la arcilla o al extracto no se vieron afectados.

Por otro lado, nos pareció interesante evaluar el contenido de los metales característicos de la composición de la arcilla en bazo de ratas expuestas a la arcilla y el extracto, no hallando diferencias notables con respecto a las ratas controles.

Los resultados obtenidos en estos experimentos han dado lugar a las siguientes publicaciones:

- ***EFFECTS OF THE SUBCHRONIC EXPOSURE TO AN ORGANOMODIFIED CLAY MINERAL FOR FOOD PACKAGING APPLICATIONS ON WISTAR RATS.*** *(Maisanaba y col., 2014; Applied Clay Science 95, 37-40).*
- ***IN VIVO EVALUATION OF ACTIVITIES AND EXPRESSION OF ANTIOXIDANT ENZYMES IN WISTAR RATS EXPOSED FOR 90 DAYS TO A MODIFIED CLAY.*** *(Maisanaba y col., 2014; Journal of Toxicology and Environmental Health, Part A: Current Issues 77,456-466).*
- ***IN VIVO TOXICITY EVALUATION OF THE MIGRATION EXTRACT OF AN ORGANOMODIFIED CLAY-POLY(LACTIC) ACID NANOCOMPOSITE.*** *(Maisanaba y col., 2014; Journal of Toxicology and Environmental Health, Part A: Current Issues 77,731-446).*
- ***EVALUACIÓN DE LA SEGURIDAD DE UNA ARCILLA MODIFICADA Y SU EXTRACTO DE MIGRACIÓN EN BAZO DE RATAS WISTAR EXPUESTAS DE FORMA SUBCRÓNICA.*** *(Maisanaba y col., 2013 Revista de Toxicología 30, 125-130).*

Para la realización de esta Tesis Doctoral, la doctoranda realizó una estancia de investigación en ITENE, bajo la dirección de las Dras. Susana Aucejo y María Jordá, en la que se abordaron varios objetivos.

Durante la estancia, se llevó a cabo el desarrollo de nuevas arcillas basadas en CNa^+ modificadas con silanos, Clay3, Clay4A y Clay4B, destinadas al envasado. La caracterización de las mismas se realizó por espectroscopía de infrarrojos (FTIR), difracción de rayos X y termogravimetría (TGA). Los resultados preliminares mostraron una buena incorporación de los modificadores y mejoraron el perfil tecnológico de la arcilla original no modificada.

Posteriormente, se evaluó la toxicidad de las tres arcillas sintetizadas. Los biomarcadores estudiados incluyeron citotoxidad basal, genotoxicidad (mediante el ensayo cometa) y estrés oxidativo (producción de ERO y contenido de GSH) en las líneas HepG2 y Caco-2. Por otro lado, también se determinó mediante citometría de flujo el posible mecanismo de muerte celular de células Caco-2 expuestas a Clay4A y Clay4B. Además, se evaluó el potencial mutagénico de las arcillas mediante el Test de Ames. De forma general, los resultados preliminares dieron lugar a una ausencia de efectos tóxicos por parte de Clay3, sin embargo, tanto Clay4A y Clay4B mostraron toxicidad en la mayoría de los parámetros ensayados. Dado que Clay3 fue la arcilla con mejor perfil tecnológico y toxicológico ésta fue seleccionada para realizar ensayos de vida útil, observándose un incremento de aproximadamente 24h en comparación con el control.

Por otro lado, se valoró la migración de metales (Al, Ca, Fe, Mg y Fe) en dos simulantes diferentes, etanol al 10% e isooctano, procedentes de materiales nanocompuestos de polipropileno (PP) más Clay3 o PP-Clay4A. En todos los casos se obtuvieron diferencias con el control en alguno de los metales determinados.

Los resultados obtenidos en estos experimentos darán lugar a diferentes publicaciones, aún pendientes de envío:

- ***DEVELOPMENT, CHARACTERIZATION AND CYTOTOXICITY OF NOVEL SILANES MODIFIED CLAYS INTENDED TO PACKAGING*** (Título provisional).
- ***TOXICITY EVALUATION OF A NEW SILANE-MODIFIED CLAY AND ITS MIGRATION EXTRACT FROM A NANOCOMPOSITE INTENDED TO FOOD PACKAGING*** (Título provisional).

- ***TOXICOLOGICAL ASSESSMENT OF TWO SILANE-MODIFIED CLAYS IN HUMAN HEPATOMA CELLS AND SALMONELLA TYPHIMURIUM STRAINS*** (Título provisional).
- ***CYTOTOXICITY, OXIDATIVE STRESS AND GENOTOXICITY ASSAYS OF SILANES-MODIFIED CLAYS IN THE HUMAN INTESTINAL CELL LINE CACO-2*** (Título provisional).

En relación a los aceites esenciales y sus componentes mayoritarios, también éstos están teniendo un gran auge en la industria alimentaria, empleándose en un nuevo tipo de envasado conocido como envasado activo. En este sentido, en vez de dar lugar a mejoras tecnológicas en sí como en el caso de las arcillas, lo que se aprovecha de estos aceites y sus componentes es su potencial antioxidante y antimicrobiano natural. Sin embargo, debe ser establecido un rango de concentraciones seguro para evitar los posibles efectos contraproducentes que puedan desencadenarse por un abuso de los mismos. En primer lugar, vimos necesario realizar una contextualización del uso actual de este tipo de envasado y las propiedades antimicrobianas y antioxidantes que presentan, así como una revisión de los principales efectos citotóxicos en diferentes líneas celulares de origen humano. Dicha revisión ha dado lugar a la presente publicación:

- ***NEW ADVANCES IN ACTIVE PACKAGING INCORPORATED WITH ESSENTIAL OILS OR THEIR MAIN COMPONENTS FOR FOOD PRESERVATION.*** *(Maisanaba y col., 2015. Food Reviews International (en revisión, under revision).*

Por último, la evaluación de los componentes mayoritarios de los aceites esenciales antes de ser incorporados a los envases es también obligatoria, tal y como establece la EFSA (EFSA, 2011b). En este sentido, el aceite esencial de orégano es uno de los más utilizados por sus destacadas propiedades antimicrobianas, siendo timol y carvacrol sus dos componentes mayoritarios. Existen en la bibliografía resultados sobre sus perfiles genotóxicos obtenidos por otros autores (Azizan y Blevins, 1995; Stammati y col., 1999; Ipek y col., 2005; Buyukleyla y Recuzogullari, 2009; Llana-Ruíz-Cabello y col., 2014). Estos resultados son dispares en algunos aspectos, siendo necesaria una evaluación de su toxicidad más amplia con el fin de esclarecer el perfil genotóxico de estos dos compuestos mayoritarios. Por consiguiente nos pareció interesante llevar a cabo el ensayo de MN, y, por primera vez, el

ensayo de Linfoma de Ratón. Los resultados obtenidos no mostraron una relevancia biológica notable para ninguno de los compuestos bajo las condiciones ensayadas. Dichos resultados se reflejan en la siguiente publicación:

- ***IN VITRO GENOTOXICITY TESTING OF CARVACROL AND THYMOL USING THE MICRONUCLEUS AND MOUSE LYMPHOMA ASSAYS.*** *(Maisanaba y col., 2015; Mutation Research 784-785, 37-44).*

SUMMARY

Currently, the food industry is betting on the incorporation of natural substances to food packaging in order to increase the perdurability of food products in the market. Due to the use of these substances in this new application, the safety of consumers and the environment may be compromised due to an increased exposure, with unknown consequences. Among the natural substances selected by the food industry for this aim, clays and clay minerals are included, as well as essential oils and their major components, being the first ones (clays) the main topic of study of this Doctoral Thesis. Therefore, we decided to carry out a toxicological assessment, both *in vitro* and *in vivo*, using a battery of toxicological tests, in order to investigate the possible toxic effects that can arise after the exposure to these substances, either pure or already incorporated into the package. The toxicological information of these substances constitutes a regulatory requirement of the competent authorities before their commercialization.

In the case of clays and clay minerals, their incorporation have been shown to improve the mechanical, thermal and barrier properties of the polymer matrix, which are intimately related to the increase of the shelf-life of food. In order to contextualize the state of the art of the clays and their derivatives in the food packaging industry, as start-point to address further studies, it is important to carry out a comprehensive review of the toxicity data published in the scientific literature up to now. Taking into account the disparity of available results, a case by case toxicological evaluation is required. Different parameters may be involved in the response observed, including: (i) exposure conditions such as selected concentrations and times of exposure tested; (ii) selected experimental models; (iii) modifiers or surfactants incorporated into the clay structure, (iv) sensitivity of the assays performed, etc. This review has led to the following publication:

- ***TOXICOLOGICAL EVALUATION OF CLAY MINERALS AND DERIVED NANOCOMPOSITES: A REVIEW.*** *(Maisanaba y col., 2015; Environmental Research 138, 233-254).*

The most important route of exposure in the context of the present work is the oral route, since clays will be incorporated into a package which is going to be in contact with food, that will be further ingested by consumers. In this case, the most affected organs could be those of

the digestive system, such as the intestine, responsible for nutrients absorption, and liver, crucial in the xenobiotics biotransformation. A set of tests with the materials under study was performed in order to clarify their major toxic effects, as the available data are scarce and contradictory. The selected cell lines were a human hepatoma cell line (HepG2) and a colon adenocarcinoma cell line (Caco-2). They were exposed to several unmodified and quaternary ammonium salts-modified clays, both commercialized (Cloisite®Na^+ (CNa^+), Cloisite®20A (C20A) and Cloisite®30B (C30B)) or to clays developed by the Technological Institute of Packaging, Transport and Logistics (ITENE) Valencia (Clay1 and Clay2). The experiments performed were: basal cytotoxicity, cell morphology analysis, genotoxicity assays (comet assay and micronuclei (MN)) and oxidative stress (production of reactive oxygen species (ROS) and glutathione content (GSH)). In general, no toxicity was obtained in the case of CNa^+, C20A and Clay1, and only in the case of the first one, a significant induction of MN and some alteration of cell morphology were obtained. However, important toxic effects were observed in both cell lines after exposure to C30B and Clay2 in most of the parameters evaluated. Furthermore, it has been carried out by the first time in the HepG2 cell line the study of the influence of clays in the modulation of the gene expression of a large group of genes involved in activation/detoxification and other toxicity mechanisms. CNa^+ and Clay2 showed deregulation in different genes, but C30B and Clay1 did not. The results of these experiments have led to the following publications:

- ***IN VITRO TOXICOLOGICAL ASSESSMENT OF CLAYS FOR THEIR USE IN FOOD PACKAGING APPLICATIONS.*** *(Maisanaba y col., 2013, Food and Chemical Toxicology 37, 266-275).*
- ***GENETIC POTENTIAL OF MONTMORILLONITE CLAY MINERAL AND ALTERATION IN THE EXPRESSION OF GENES INVOLVED IN TOXICITY MECHANISMS IN THE HUMAN HEPATOMA CELL LINE HEPG2.*** *(Maisanaba y col., 2015; Journal of Hazardous Materials (en revision/ under revision)).*
- ***INDUCTION OF MICRONUCLEI AND ALTERATION OF GENE EXPRESSION BY AN ORGANOMODIFIED CLAY IN HEPG2 CELLS. (****Maisanaba y col., 2015; Archives of Toxicology (en revisión/ under revision)).*
- ***EFFECTS OF TWO ORGANOMODIFIED CLAYS INTENDED TO FOOD CONTACT MATERIALS ON THE GENOMIC INSTABILITY AND GENE***

EXPRESSION OF HEPATOMA CELLS. *(Maisanaba y col., 2015); Toxicology Letters (en revision/ under revision).*

- ***TOXIC EFFECTS OF A MODIFIED MONTMORILLONITE CLAY ON THE HUMAN INTESTINAL CELL LINE CACO-2.*** *(Maisanaba y col., 2014; Journal of Applied Toxicology 34, 714-725).*
- ***TOXICITY ASSESSMENT OF ORGANOMODIFIED CLAYS USED IN FOOD CONTACT MATERIALS ON HUMAN TARGET CELL LINES.*** *(Houtman y col., 2014; Applied Clay Science 90, 150-158).*

Furthermore, although the organs and tissues of the digestive system would probably be the most affected by the exposure to clays, other targets could also be damaged, highlighting the vascular endothelial tissue, involved in distribution and absorption. In this concern, data on the effects of this experimental model after exposure to clays are limited so far. Therefore, cell viability evaluation of human umbilical vein endothelial cells (HUVEC) exposed to CNa^{+}, C30B, Clay1 and Clay2 was carried out. The results showed that HUVEC response followed a similar pattern than HepG2 and Caco-2 upon exposure to clays, with a sensitivity close to the hepatic cell line. In addition, the study of the mutagenic potential using the Ames test is required before marketing any product that come into contact with food (EFSA 2011b, 2015), such as clays, which are intended to be used in food packaging. The experimental model selected was *Salmonella typhimurium* (5 strains), which were exposed to the four mentioned clays, in the absence and presence of external metabolic activation. We only observed a positive mutagenic response in the case of C30B and Clay1. The results obtained are compiled in the following publication:

- ***CYTOTOXICITY AND MUTAGENICITY ASSESSMENT OF ORGANOMODIFIED CLAYS POTENTIALLY USED IN FOOD PACKAGING.*** *(Maisanaba y col., 2015; Toxicology In Vitro 29, 1222-1230).*

The European Food Safety Authority (EFSA) proposes, not only the evaluation of materials that are incorporated into packaging polymer matrices, but also the evaluation of the resulting packages (EFSA, 2011a, b). Thus, ITENE developed two nanocomposites based on poly (lactic acid) (PLA) and Clay1, and PLA-Clay2, both modified clays designed by them. With these materials migration tests were carried out. The food stimulant used was distilled

water, accepted by the EU Regulation No. 10/2011, due to the type of food products these nanocomposites will be used for (hydrophilic food).

Once the migration extracts of PLA-Clay1 and PLA-Clay2 were obtained, several parameters were evaluated in order to determine the potential toxic effects induced after their exposure. Cell viability of HepG2 and Caco-2 exposed to both extracts and the mutagenic potential by the Ames test were evaluated. The results shown an absence of cytotoxicity and mutagenicity under the conditions tested. Moreover, considering that the main metals present in clays structure are Al, Ca, Fe, Mg and Si, their contents in the migration extracts were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). No significant differences with respect to the control group were found. These results are summarized in the following publication:

- ***CYTOTOXICITY AND MUTAGENICITY STUDIES ON MIGRATION EXTRACTS FROM NANOCOMPOSITES WITH POTENTIAL USE IN FOOD PACKAGING.*** *(Maisanaba y col., 2014 Food and Chemical Toxicology 66, 366-372).*

Once the *in vitro* toxicology studies were conducted, the clay with the best technological properties and toxicological profile was selected for further experiments, Clay1. In order to complete the *in vitro* results and taking into account that *in vivo* data are scarce, a repeated dose 90-day oral toxicity study in Wistar rats exposed to Clay1 (40 mg/kg/day) with the diet and its migration extract (PLA-Clay1) as drinking water (*ad libitum*) was performed. In this sense, after the exposure period, the animals were sacrificed and their organs (liver, kidney, intestine, brain, heart, testicles, lungs and spleen) and blood by cardiac puncture were removed. Several parameters were evaluated, including: histopathological analysis, clinical biochemistry of blood serum, interleukin 6 (IL-6) leackage, biomarkers involved in oxidative stress response in liver and kidney, such as reduced/oxidized glutathione ratio (GSH/GSSG), lipid peroxidation (LPO), and activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-Transferase (GST). Moreover, gene expression and protein abundance of SOD and CAT in liver and kidney were also evaluated. We can highlight an increase of CAT activity in kidney, as well as an alteration in gene expression and protein abundance after exposure to Clay1. All these biomarkers remained unaltered after the exposure to PLA-Clay1 migration extract.

In addition, it was interesting to evaluate the content of the typical metals presented in clays composition in the spleen of exposed rats. Significant differences were not observed compared to control rats.

The results obtained in these experiments have led to the following publications:

- ***EFFECTS OF THE SUBCHRONIC EXPOSURE TO AN ORGANOMODIFIED CLAY MINERAL FOR FOOD PACKAGING APPLICATIONS ON WISTAR RATS.*** *(Maisanaba y col., 2014; Applied Clay Science 95, 37-40).*
- ***IN VIVO EVALUATION OF ACTIVITIES AND EXPRESSION OF ANTIOXIDANT ENZYMES IN WISTAR RATS EXPOSED FOR 90 DAYS TO A MODIFIED CLAY.*** *(Maisanaba y col., 2014; Journal of Toxicology and Environmental Health, Part A: Current Issues 77,456-466).*
- ***IN VIVO TOXICITY EVALUATION OF THE MIGRATION EXTRACT OF AN ORGANOMODIFIED CLAY-POLY(LACTIC) ACID NANOCOMPOSITE.*** *(Maisanaba y col., 2014; Journal of Toxicology and Environmental Health, Part A: Current Issues 77,731-446).*
- ***EVALUACIÓN DE LA SEGURIDAD DE UNA ARCILLA MODIFICADA Y SU EXTRACTO DE MIGRACIÓN EN BAZO DE RATAS WISTAR EXPUESTAS DE FORMA SUBCRÓNICA.*** *(Maisanaba y col., 2013 Revista de Toxicología 30, 125-130).*

For the fulfillment of this thesis, the PhD student performed an internship in ITENE, under the direction of Drs. Susana Aucejo and Maria Jordá, where different objectives were addressed.

During the internship, new silanes-modified clays based in CNa^{+}, Clay3, Clay4A and Clay4B, were developed intended to food packaging. The characterization of these clays was performed by infrared spectroscopy (FTIR), X-ray diffraction and thermogravimetric analysis (TGA). Preliminary results showed good incorporation of modifiers and improved technological profile in comparison to the raw clay.

Subsequently, a toxicological evaluation of the three synthesized clays was performed. Biomarkers studied included basal cytotoxicity, genotoxicity (by the comet assay) and oxidative stress induction (production of ROS and GSH content) in HepG2 and Caco-2 cell

lines. Furthermore, the possible mechanism of cell death of Caco-2 cells exposed to Clay4A and Clay4B was studied by flow cytometry. In addition, the mutagenic potential of clays was evaluated by the Ames test. In general, the preliminary results led to an absence of toxic effects by Clay3; however, both Clay4A and Clay4B showed toxicity in almost all parameters tested. In this sense, Clay3 showed the best technological and toxicological profile. Therefore, it was selected for food shelf- life testing, giving an increase of approximately 24 hours compared with the control.

Moreover, the migration of metals (Al, Ca, Fe, Mg and Fe) from nanocomposites of polypropylene (PP)-Clay3 or PP-Clay4A was evaluated. In all cases differences from controls in some of the specific metals studied were obtained.

The results of these experiments will be included in different publications, pending to be send:

- ***DEVELOPMENT, CHARACTERIZATION AND CYTOTOXICITY OF NOVEL SILANES MODIFIED CLAYS INTENDED TO PACKAGING*** (provisional title).
- ***TOXICITY EVALUATION OF A NEW SILANE-MODIFIED CLAY AND ITS MIGRATION EXTRACT FROM A NANOCOMPOSITE INTENDED TO FOOD PACKAGING*** (provisional title).
- ***TOXICOLOGICAL ASSESSMENT OF TWO SILANE-MODIFIED CLAYS IN HUMAN HEPATOMA CELLS AND SALMONELLA TYPHIMURIUM STRAINS*** (provisional title).
- ***CYTOTOXICITY, OXIDATIVE STRESS AND GENOTOXICITY ASSAYS OF SILANES-MODIFIED CLAYS IN THE HUMAN INTESTINAL CELL LINE CACO-2*** (provisional title).

Regarding to the essential oils and their main components, they are also having a boom in the food industry, being used in a new type of packaging known as active packaging. In this case, the technological improvements are due to the antioxidant and antimicrobial natural properties presented by this kind of substances. However, it should be established a safe range of use to avoid the possible adverse effects due to their abuse. First, we carried out a contextualization of the current use of this type of packaging and the antimicrobial/ antioxidant properties they showed, and also, a review of their main cytotoxic effects in different cell lines of human origin. That review has resulted in this publication:

- ***NEW ADVANCES IN ACTIVE PACKAGING INCORPORATED WITH ESSENTIAL OILS OR THEIR MAIN COMPONENTS FOR FOOD PRESERVATION.*** *(Maisanaba y col., 2015. Food Reviews International (en revisión, under revision).*

Finally, the evaluation of the major components of the essential oils before their incorporation to the packaging is also mandatory, as established by the EFSA (EFSA, 2011b). In this regard, oregano essential oil is one of the most used for its outstanding antimicrobial properties, being thymol and carvacrol their two main components. In the literature, there are several data about their genotoxic profiles (Azizan and Blevins, 1995; Stammati et al., 1999; Ipek et al., 2005; Buyukleyla and Recuzogullari, 2009; Llana-Ruiz-Cabello et al, 2014). These results are inconclusive in some aspects, so a comprehensive assessment of thymol and carvacrol, focussed in their genotoxic profile, is necessary. Therefore, it was interesting to conduct the MN test and, for the first time, the mouse lymphoma assay. The results showed no significant biological relevance for any of the compounds under the conditions tested. These results are described in the following publication:

- ***IN VITRO GENOTOXICITY TESTING OF CARVACROL AND THYMOL USING THE MICRONUCLEUS AND MOUSE LYMPHOMA ASSAYS.*** *(Maisanaba y col., 2015; Mutation Research 784-785, 37-44).*

II. INTRODUCCIÓN / INTRODUCTION

1. ORIGEN DE LAS ARCILLAS Y MINERALES DE ARCILLA

Las arcillas y minerales de arcilla (también conocidos como láminas de silicatos) pertenecen al grupo de filosilicatos (del griego "*phyllon*": hoja, y del latín "*silic*": piedra) (Meunier, 2005). Estos materiales se caracterizan por ser muy pequeños (un tamaño máximo de micrómetros), pudiéndolos encontrar de forma de forma natural, provenientes de rocas o cenizas volcánicas, bajo superficie (suelos y sedimentos), como es el caso de las arcillas, o bien, como resultado de la meteorización química (diagénesis y alteraciones hidrotermales) de otros minerales de silicato, en el caso de los minerales de arcilla (Meunier, 2005; Bergaya y Lagaly, 2006; Choy y col, 2007; Floody y col, 2009). A pesar de presentar características muy similares, existen varios aspectos que diferencian a las arcillas y minerales de arcilla que se exponen en la **Tabla 1.**

Arcilla	**Minerales de Arcilla**
Natural	Natural o sintética
Grano fino (<2-4 μm)	No hay criterio establecido para el tamaño
Filosilicatos como principales constituyentes	Pueden no incluirse filosilicatos en su estructura
Plásticas (con excepción de arcillas con origen pedroso)	Plásticas
Se endurece con el secado o cocción	Se endurece con el secado o cocción

Tabla 1. Diferencias entre arcillas y minerales de arcillas (tomada de Bergaya y Lagaly, 2006).

2. CLASIFICACIÓN Y PRINCIPALES CARACTERÍSTICAS DE ARCILLAS Y MINERALES DE ARCILLA

Las propiedades físicas y químicas de las arcillas y minerales de arcillas dependen de su estructura y composición, pudiendo clasificar de tal forma un amplio grupo según las características presentadas (**Fig. 1**).

Las arcillas son materiales conformados en láminas de tetraedros de silicato (SiO_4) y octaedros (conteniendo Al, Mg y Fe). Estos minerales arcillosos naturales se construyen basados en unidades estructurales laminadas con un espesor individual de cada lámina de aproximadamente uno a unos pocos nanómetros y dimensiones laterales

que van desde 30 nm a varios micrómetros, obteniendo una relación de longitud - espesor superior a 1000 nm (Zhu y Njuguna, 2014).

De acuerdo con su composición química, características de la estructura y la carga neta, podemos clasificar siete grupos: (1) caolinita-serpentina, (2) talco- pirofilita, (3) esmectita, (4) vermiculita, (5) mica, (6) clorita, y (7) arcillas interestratificadas (Martin y col., 1991).

El grupo más simple presenta una estructura con una relación 1:1, en el que una capa tetraédrica de sílice se fusiona por compartición de átomos de oxígeno a un octaedro de aluminio (por ejemplo, caolinita). El siguiente grupo presenta una estructura con relación 2:1, conocido generalmente como el grupo de los filosilicatos, el cual consta de una lámina octaédrica central de aluminio ubicada entre dos láminas tetraédricas de sílice. Las unidades laminadas del silicato, a menudo conocidas como estructura interlaminar o galería laminar, se encuentran posicionadas en paralelo y están unidas por fuerzas de Van der Waals y electrostáticas. La sustitución isomórfica dentro de las láminas genera cargas negativas que normalmente se ven contrarrestadas por sodio o calcio presentes en el espacio interlaminar. Estos cationes intercambiables pueden ser reemplazados por cationes orgánicos e inorgánicos a través de reacciones de intercambio iónico. Dentro de la familia de los filosilicatos 2:1 se encuentran los silicatos más ampliamente conocidos en el mundo científico y usados por la industria, las esmectitas (Bignon, 1990; Gilman, 1999; Williams y col., 2004; Goettler y col., 2007; Leszczynska y col., 2007a, 2007b; Drummy y col. ,2010; Garrido-Ramírez y col., 2010; Gatica y Vidal, 2010; Kiliaris y Papaspyrides, 2010; Yamada y col., 2011).

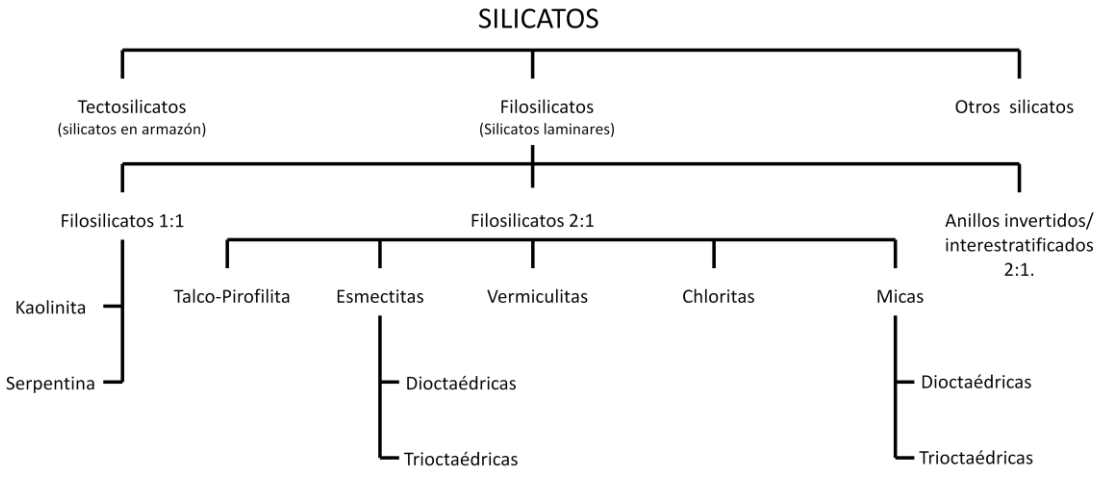

Figura 1. Clasificación de silicatos y principales subgrupos de arcillas (tomada de OMS, 2005).

En el grupo de las esmectitas, una de las más relevantes es la Montorillonita (Mt). Las esmectitas están compuestas por una capa octaédrica custodiada por dos láminas opuestas tetraédricas, siendo la estructura general de este tipo de filosilicatos (Leszczynska y col, 2007a, 2007b; Theng, 2012) (**Fig. 2**). La molécula de Si^{4+} del tetraedro de SiO_4 está dispuesta entre cuatro O^{2-} para conformar una red con estructura hexagonal, compartiendo tres esquinas con tetraedros adyacentes. Cada octaedro que forma la arcilla (p.ej. AlO_6 o MgO_6) en la capa octaédrica está conectado a octaedros vecinos mediante los bordes de los mismos, en los cuales están presentes grupos hidroxilos, formando una estructura hexagonal o pseudohexagonal simétrica. La carga neta de la esmectita es negativa debido a la sustitución isomorfa de iones de silicio y otros metales de baja carga (p.ej. Al^{3+} sustituido por Mg^{2+} y Fe^{2+}, o Mg^{2+} sustituido por Li^{1+}). Las cargas negativas son contrarrestadas por cationes alcalinos y alcalinotérreos presentes entre las dos láminas tetraédricas dispuestas una frente a otra. Los cationes intercambiables son hidratados, entrando el agua o solvente en la capa intermedia de la estructura y ampliando el armazón de la arcilla, pudiendo acomodar compuestos adicionales, incluyendo especies con carga neutra y/o negativas. Debido a la capacidad única de la esmectita para incorporar diversos iones, la superficie de la misma puede ser hidrófila o hidrófoba dependiendo de la carga y solutos disponibles.

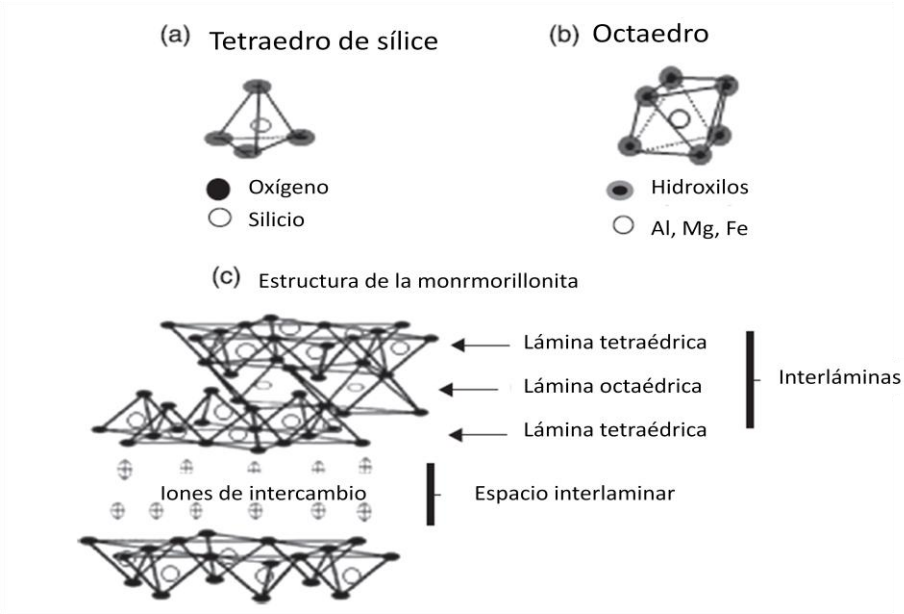

Figura 2. Representación esquemática de la estructura de la Mt. a) tetraedro de sílice coordinado por cuatro O^{2-}; b) octaedro en el cual el ión central está coordinado por seis hidroxilos; c) estructura de la Mt, indicando los componentes de la unidad interlaminar y el espacio interlaminar (tomada de Zhu y Njuguna, 2014).

Debido a la relativa alta capacidad de intercambio catiónico, alto ratio dimensional que presentan, y a la facilidad de expansión del espacio de capa interlaminar, las esmectitas pueden interactuar con una amplia gama de especies orgánicas, siendo una propiedad crítica requerida para muchas aplicaciones llevadas a cabo en varios sectores de la industria (Zhu y Njuguna, 2014).

3. USOS GENERALES DE LAS ARCILLAS Y MINERALES DE ARCILLA

Como materiales naturales, ecológicos, abundantes, de bajo costo y altamente reactivos, las arcillas se han utilizado desde hace muchos años para una amplia variedad de propósitos en nuestra sociedad, y actualmente se les conoce como los uno de los materiales en la industria de minerales de los cuales se pueden sacar mayor provecho, conociéndose como los materiales del siglo XXI (Bergaya y Lagaly, 2013; Zhu y Njuguna, 2014). A día de hoy, podemos encontrar en bibliografía más de 100 aplicaciones industriales de materiales de arcilla. Cabe destacar su uso en el sector de las industrias de procesado, en industrias agrícolas para el uso en la nutrición de animales de granja como agentes y aditivos de unión para piensos, con el fin de promover el crecimiento y la salud de los animales, además de como suplemento para el tratamiento de trastornos gastrointestinales, en la industria de la ingeniería y construcción, para remedios ecológicos y geología, en medicina como transportadores de fármacos, y otras tantas aplicaciones industriales (Murray y col., 2007; Baek et al., 2012). Es también muy importante mencionar que en la última década, motivado también por el auge de la nanotecnología, el interés por los materiales que nos competen ha aumentado considerablemente. Como curiosidad, comentar que se han publicado más de 10000 artículos científicos considerando todas las áreas de aplicación mencionadas anteriormente, principalmente en los años 2008-2009 (Zhu y Njuguna, 2014).

Actualmente, como veremos a lo largo de esta tesis doctoral, la apuesta por el uso de arcillas, sobre todo las ya mencionadas Mt y caolinita, en la industria alimentaria, y más específicamente en el área de envasado de alimentos, es una realidad, dando lugar a una mejora del envasado tradicional frente al envasado modificado.

4. ENVASADO ALIMENTARIO Y ARCILLAS: MATERIALES NANOCOMPUESTOS

Como se ha comentado anteriormente, la industria alimentaria y más concretamente el área de envasado de alimentos, está apostando a día de hoy por el uso de un nuevo tipo de envasado, el cual va íntimamente ligado al uso de arcillas, destacando entre ellas la Mt y la caolinita, gracias a las propiedades y características que ellas presentan, y a la ciencia que a día de hoy está en auge, la nanotecnología. En el contexto de envases de alimentos y bebidas, el uso de la nanotecnología para introducir mejoras a la propiedad de materiales se refiere en gran parte a la introducción de nanorellenos en polímeros tradicionales para generar nuevos compuestos poliméricos (Plackett y Siró, 2012). Entre estos posibles nanorellenos se encuentran las arcillas y minerales de arcillas, generalmente considerados como nanomateriales, ya que poseen, como hemos descrito previamente, láminas con un grosor nanométrico, pudiéndose de tal forma nombrarlas indistintamente como arcillas o nanoarcillas (Zhu y Njuguna, 2014). Por esta característica y por todas las ventajas comentadas en los apartados anteriores es por lo que se destacan sus usos en éste área, dando lugar a materiales novedosos con características mejoradas.

4.1. POLÍMEROS NANOCOMPUESTOS: QUÉ SON Y QUÉ VENTAJAS PRESENTAN

Los polímeros nanocompuestos se pueden definir como materiales poliméricos que contienen nanorellenos bien dispersados en forma de partículas, fibras, láminas u otros elementos, que exhiben una mejora notable en una serie de características comparado con el polímero tradicional, incluyendo las propiedades mecánicas, propiedades de barrera, estabilidad térmica, y retardo en la ignición, entre otras (Plackett y Siró, 2012). En el caso de los polímeros nanocompuestos obtenidos por la incorporación de arcillas, sobre todo Mt, surgen por la adición de pequeñas cantidades de arcillas o minerales de arcillas a la matriz de un polímero tradicional, obteniéndose un material final mejorado destinado a enriquecer el fin último del polímero convencional, incrementar la vida útil en el mercado de los alimentos perecederos (Annabi-Bergaya, 2008).

Como hemos descrito anteriormente, la Mt cuenta con una estructura multilaminar. Cada una de las láminas que componen ese conjunto presenta dos capas tetraédricas que acogen a una capa central octaédrica, siendo la suma de las tres de tamaño nanométrico con una alta relación dimensional (Uyama y col., 2003), frente al tamaño micrométrico que presentaría la partícula completa. Cuando las arcillas son dispersadas en la matriz polimérica, experimentan una hinchazón haciendo que todas las láminas que forman la partícula se separen, dividiéndose individualmente en unidades con grosor nanométrico, obteniéndose a partir de este momento el ya nombrado polímero nanocompuesto.

La separación de las plaquetas de arcilla obligan a los gases a seguir un camino tortuoso a través del material que reduce en gran medida su transmisión y llegada al alimento (**Fig. 3**). Como resultado podríamos decir que se obtiene una estructura de arcilla dispuesta en nanocapas aumentándose el camino de difusión de las moléculas externas y el tiempo de penetración de gases y otras sustancias, proporcionando productos sustancialmente mejorados (Abacha y col.,2009; Choudalakis y Gotsis, 2009; Herrera- Alonso y col., 2010).

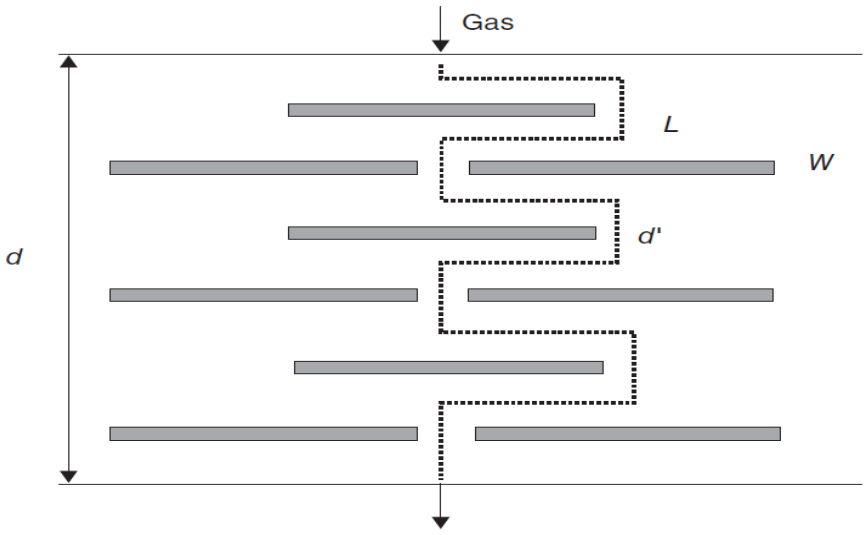

Figura 3. Representación esquemática que explica cómo las nanoarcillas pueden dar lugar a un camino tortuoso hasta llegar al alimento que influye en la capacidad de permeabilidad de los gases a través de la matriz polimérica (d: espesor de la membrana polimérica; *d´*: trayectoria/longitud de la difusión del gas en presencia de las nanoláminas de arcillas; *L*: espesor de las nanoláminas de arcilla; W: grosor de las nanoláminas de arcilla).

El efecto de las nanoarcillas en las propiedades de la matriz polimérica se centra fundamentalmente en la alta relación superficie-volumen, ya que las interacciones polímero-relleno están principalmente gobernadas por las fuerzas presentes en la estructura interlaminar (Arora y Padua, 2010), descritas anteriormente en el apartado 2.

4.2. ELABORACIÓN DE UN MATERIAL NANOCOMPUESTO POLIMÉRICO DE ARCILLA

Existen diferentes métodos para la preparación de una material nanocompuesto polimérico de arcilla: a) Intercalación por fusión/fundición, b) polimerización *in situ* y c) dispersión por solución. El método de intercalación por fusión es el seleccionado en mayor medida por la industria debido a la naturaleza continua del proceso, por tener consecuencias poco severas para el medio ambiente, y, por los factores económicos (Zhu y Njuguna, 2014). Esta técnica consiste en mezclar el silicato estratificado con la matriz polimérica en estado fundido. Si se cuenta con una alta compatibilidad entre las láminas de las arcillas y el polímero, las primeras pueden quedar perfectamente imbuidas en la matriz, penetrando el polímero en el espacio interlaminar (Pavlidou y Papaspyrides, 2008).

Sin embargo, la formación exitosa de un material nanocompuesto polimérico de arcilla, además de en la buena elección del método de preparación, se basa en dos características clave del componente de refuerzo, la arcilla. La primera característica, presente en el grupo de las esmectitas como ya se ha comentado, es la habilidad de poder modificar la superficie química de los silicatos mediante reacciones de intercambio iónico con cationes orgánicos e inorgánicos con el fin de producir un "nanocomponente" compatible con el polímero. La segunda característica es la capacidad de dispersión de las partículas de silicato en la matriz polimérica, dando lugar a un material nanocompuesto exfoliado. En este sentido, podemos encontrar tres disposiciones diferentes de arcilla tras su incorporación: 1) disposición en bloque, dándose cuando el espacio interlaminar de la estructura o galería laminar no se expande, debido, por lo general, a la pobre afinidad con el polímero. No se conocen por el momento polímeros nanocompuestos con esta disposición (Alexandre y Dubois, 2000). 2) disposición intercalada de las láminas de la arcilla, obteniéndose una moderada expansión de la capa intermedia de arcilla. En este caso, los espacios entre capas se

expanden ligeramente a medida que las cadenas de polímero penetran el espaciamiento basal de la arcilla, pero la forma de bloque en capas permanece. Este es el resultado de una afinidad moderada entre el polímero y la arcilla. Y por último, 3) disposición dilatada de las estructuras laminares, las partículas de arcilla pierden su identidad en capas y están bien separados en láminas sueltas dentro de la matriz. Esto se debe a una gran afinidad entre el polímero y arcilla (Arora y Padua, 2010) (**Fig. 4**). Estas dos características se encuentran, por supuesto, conectadas entre sí debido a que el grado de dispersión de las láminas de un silicato matriz polimérica depende de la modificación de cationes capa intermedia (Ray y Okamoto y col., 2003).

Sin embargo, una dispersión simple de arcillas, dígase Mt, en una matriz polimérica, no va a producir un material nanocompuesto con destacadas mejoras, debido a las pobres interacciones interfaciales entre los sitios de reacción hidrófilos de la arcilla y la alta hidrofobicidad con la que cuentan las cadenas del polímero (Pisticelli y col. , 2010). En este sentido, la solución para esta notable desventaja se puede solventar mediante la introducción de grupos orgánicos para mejorar la interacción entre las arcillas y polímeros (Silva y col., 2011).

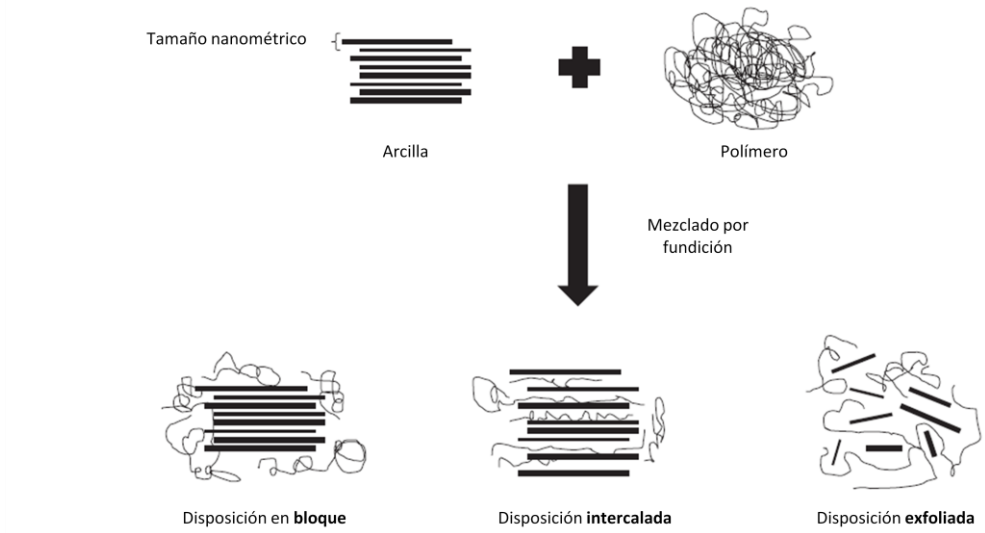

Figura 4. Representación esquemática de la posible disposición de la arcilla para conformar el polímero nanocompuesto (tomada de Arora y Padua, 2010, con modificaciones).

4.3. ARCILLAS Y MODIFICADORES ORGÁNICOS

Como se ha expuesto en el apartado anterior, debido al bajo grado de exfoliación que presentan las arcillas en la matriz polimérica por la naturaleza hidrofílica con la que cuentan y el carácter hidrófobico presentado por las cadenas del polímero, el sector industrial lleva a cabo una serie de modificaciones en la estructura de las mismas con el fin de mejorar este aspecto.

Los métodos llevados a cabo para conseguir una arcilla más organofílica son varios, destacando principalmente a) la reacción de intercambio iónico (*ion exchange reaction*) y b) la reacción de injerto (*grafting reaction*).

a. Reacción de intercambio iónico

La reacción de intercambio iónico, un proceso de modificación química ampliamente utilizado para dar lugar a arcillas más organofílicas, se define como una reacción reversible en la que los cationes se intercambian estequiométricamente entre una solución electrolítica y la fase superficial del material intercambiador (Bladel y Laudelout, 1996; Pisticelli y col., 2010), en nuestro caso, la arcilla.

Cabe considerar a las arcillas como polielectrolitos multivalentes ideales para este tipo de reacciones. El exceso de carga negativa de las capas de silicatos y su capacidad de intercambio de iones se puede cuantificar mediante una específica propiedad conocida como la capacidad de intercambio catiónico (CIC), expresada en mequiv./g (Alexandre y Dubois, 2000; Manias y col., 2001). En el caso de las esmectitas, grupo de filosilicatos con mayor CEC (0.7-1.2 moles/kg) (Sherman, 2011), cuentan con una capa de intermedia de cationes hidratados, pudiendo ser desplazados por otros grupos orgánicos mediante una solución acuosa dando lugar a la modificación en su composición y por tanto obteniendo una arcilla modificada con características mejoradas de compatibilidad (Sherman, 2011). Más específicamente, la Mt sódica (CIC 110 mequiv./100g (Ray y Okamoto, 2003)), formadapor dos capas tetraédricas constituidas por átomos de Si^{4+} y O^{2-}, y, fusionadas con una capa octaédrica con átomos Al^{3+} y Mg^{2+} unidos a grupos oxígeno e hidroxilo, es ampliamente utilizada en este tipo de modificaciones. Debido a una sustitución isomórfica, el Al^{3+} puede ser sustituido por el Mg^{2+}, creando un exceso de carga negativa, que se equilibra por cationes (p. ej. Na^{+}, Ca^{2+}) colocado en el espacio entre la capa intermedia plaquetas (Jordá-Beneyto y col.,

2014). Para la mejora de la miscibilidad e interacción de las láminas de silicato entre esta arcilla y el polímero, uno debe convertir la superficie hidrófila del silicato a una organófila. Generalmente, esto se lleva a cabo gracias a la reacción de intercambio iónico mediante la incorporación de tensioactivos catiónicos como modificadores. Entre los modificadores más utilizados para obtener estas organoarcillas encontramos los cationes cuaternarios de alquilamonio, comúnmente conocidos como sales de amonio cuaternario, siendo los más destacados en la industria del envasado alimentario (**Fig. 5**), junto con los cationes alquilofosfonio. Estos cationes modificadores, una vez que son incorporados, reducen la energía superficial de las láminas de arcilla y mejoran las características de humectación de la matriz polimérica, dando como resultado un mayor espaciamiento de las mismas. Además, estos modificadores pueden proporcionar grupos funcionales que pueden reaccionar con la matriz polimérica, o, en algunos casos, inician la polimerización de monómeros para mejorar la resistencia entre la interfase inorgánica y la matriz polimérica orgánica (Blumstein, 1965; Krishnamoorti y col., 1996).

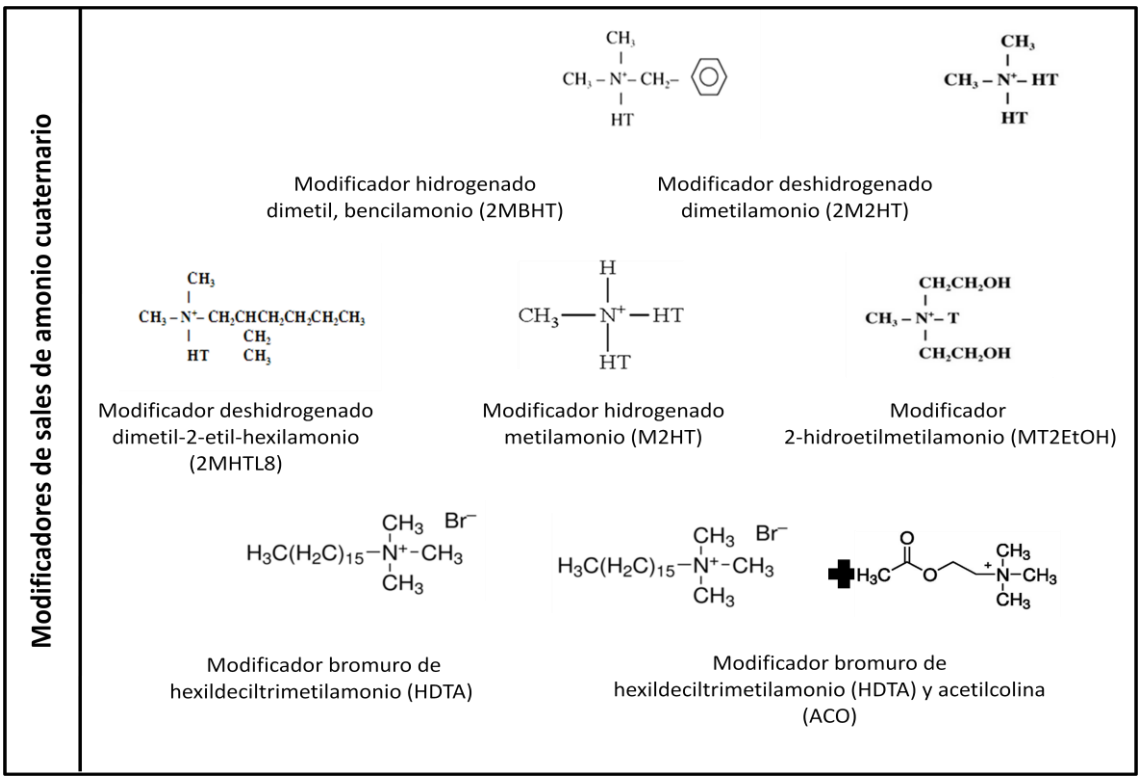

Figura 5. Modificadores comerciales de sales de amonio cuaternario más usados en arcillas modificadas.

La mejora por la incorporación de arcillas modificadas con sales de amonio cuaternarias a polímeros bien conocidos está más que corroborada. Por ejemplo, Fukushima y col. (2012) describieron como dos tipos de nanocompuestos de ácido poliláctico (PLA), polímero orgánico y biodegradable (Jamshidian y col., 2010), uno con un 5% de una Mt modificada orgánicamente, Cloisite®20A (C20A), y otro con sepiolita no modificada, presentaron una mejora tras la incorporación de las arcillas, siendo más destacadas en aquel basado en la Mt modificada. Ambos tipos de nanocompuestos dieron lugar a propiedades térmicas y de viscosidad mejoradas Además, no se afectó la transparencia del polímero tras la incorporación de las partículas. Sin embargo, la permeabilidad del plástico únicamente tuvo una variación significativa en el caso del nanocompuesto de PLA-C20A.

b. Reacción de inserción

Debido a la degradación de las sales de amonio cuaternario a altas temperaturas a través de la reacción de Hoffman, la inestabilidad térmica de la arcilla orgánica se convierte en una notable limitación en el procesamiento de nanocompuestos poliméricos de arcilla modificadas con estos tensioactivos (Xie y col, 2001, 2002; Wang y col, 2012).

Por esta razón, la reacción de injerto de grupos hidrófobos en las superficies de la capa ha supuesto una alternativa alcanzable. Esta reacción de injerto, se lleva a cabo generalmente entre los agentes de acoplamiento de silanos y los grupos silanoles reactivos que se encuentran en los bordes de las plaquetas de arcilla y en los defectos estructurales situados en la superficie de la capa intermedia y externa (Di Gianni y col., 2008; Herrera y col., 2010; Pisticelli y col., 2010). En resumen, la reacción de injerto es una solución irreversible para obtener arcilla modificadas químicamente en las que las moléculas del modificador silano (**Fig. 6**) están unidas a las capas de la arcilla seleccionada mayoritariamente, en este caso Mt (Wypych, 2004). Varios autores han investigado la modificación de arcillas con silanos incorporados a diferentes polímeros teniendo en cuenta los diferentes métodos de modificación y la obtención de varios resultados, la mejora o la disminución de las propiedades en comparación con la arcilla sin modificar y los polímeros a granel (Di Gianni y otros, 2008; Shen y col., 2007; Pisticelli y col., 2010; Silva y col., 2011).

Figura 6. Silanos utilizados como modificadores de arcillas.

En la actualidad, en la industria alimentaria podemos encontrar arcillas organomodificadas con estos tipos de modificadores ya patentados y comercializados, y otras tantas, bajo desarrollo y evaluación. El fin último es dar lugar a una arcilla con el mejor perfil tecnológico y toxicológico, con el objetivo de obtener el nanocompuesto polimérico de arcilla con las mejores propiedades para incrementar la vida útil del alimento y que sea seguro para el consumidor. Las principales arcillas comercializadas y en desarrollo se muestran en la **Tabla 2**.

Nombre	**Composición química**	
	Modificador orgánico	**Concentración del modificador**
Cloisite®Na^+ (CNa^+, comercial)	No modificada (Mt natural)	No presenta
Cloisite®30B(C30B, comercial)	Mt modificada con sal de amonio cuaternario (MT2EtOH)	90meq/100g clay
Cloisite®20A(C20A, comercial)	Mt modificada con sal de amonio cuaternario (2M2HT)	95meq/100g clay
Cloisite®93A(C93A, comercial)	Mt modificada con sal de amonio terciario (M2HT)	90meq/100g clay
Cloisite®15A(C15A, comercial)	Mt modificada con sal de amonio cuaternario (2M2HT)	125meq/100g clay
Cloisite®10A(C10A, comercial)	Mt modificada con sal de amonio cuaternario (2MBHT)	125meq/100g clay
Clay1(en desarrollo)	Mt modificada con sal de amonio cuaternario (HDTA)	6 veces del CEC de la Mt, CEC de Mt= 92.6 meq/100g
Clay2(en desarrollo)	Mt modificada con sal de amonio cuaternario (HDTA+ACO)	HDTA en 5.75 y ACO en 0.25 veces del CEC

		de Mt , CEC de Mt = 92.6meq/100g
Clay3(en desarrollo)	Mt modificada con silano (3-Aminopropiltrietoxisilano)	4 veces del CEC de la Mt
Clay4A(en desarrollo)	Mt modificada con silano (Viniltrimetoxisilano)	4 veces del CEC de la Mt
Clay4B(en desarrollo)	Mt modificada con silano (Viniltrimetoxisilano)	8 veces del CEC de la Mt

Tabla 2. Principales arcillas comerciales y en desarrollo destinadas a la industria alimentaria.

5. EVALUACIÓN TOXICOLÓGICA DE ARCILLAS NO MODIFICADAS, ORGANOARCILLAS Y MATERIALES NANOCOMPUESTOS.

Teniendo en cuenta que los materiales nanocompuestos poliméricos de arcilla están teniendo una gran potencialidad de aplicaciones en el sector del envasado de alimentos como un innovador material de embalaje con propiedades funcionales nuevas que dan lugar a la protección y mejor calidad de los alimentos. Se hace necesario evaluar la seguridad hacia los consumidores, ya que estamos ante materiales en contacto con alimentos que posteriormente serán ingeridos (Rhim y col., 2013).

Aunque los efectos tecnológicos son conocidos y están generalmente bien descritos desde hace años, los potenciales efectos toxicológicos y el impacto de las arcillas sin modificar o modificados y de los nanocompuestos poliméricos derivados sobre la salud humana y el medio ambiente, están actualmente siendo investigados exhaustivamente con mayor interés. Hay que tener en cuenta que la exposición de la población en general a bajas concentraciones de Mt, caolinita y otros minerales de arcilla en su forma natural es ubicua (OMS, 2005).

Respecto a la incorporación de las arcillas a los polímeros empleados en la industria alimentaria puede resultar como una exposición no intencionada a ellas para el consumidor, por lo que es necesario no sólo evaluar la arcilla microestructurada modificada o no, sino también el nanocompuesto resultante una vez imbuida ésta, debido a la posible migración al producto alimenticio (EFSA, 2011a; Guillard y col, 2010; Song y col., 2011; EFSA, 2015).

Es razonable asumir que esta migración se produce a causa de las pequeñas dimensiones que presentan estos nanocomponentes. También, hay indicios de que la migración podría estar relacionada con el desgaste en el tiempo del film/plástico (Rejinders, 2006). En consecuencia, la principal preocupación sobre la aplicación de nanocompuestos poliméricos usados en envasado en contacto con alimentos está relacionada con la exposición indirecta debido a la potencial migración del envase al alimento. Por ello, para los consumidores, la primera preocupación es verificar la medida de la migración de las nanoarcillas del film al alimento, y, posteriormente, si esto ocurre, a qué efectos pueden dar lugar desde que las ingieren hasta el final del tracto gastrointestinal.

Actualmente, en relación a las arcillas, modificadas o no, podemos encontrar varios trabajos sobre la evaluación toxicológica de arcillas ya comerciales utilizadas en envasado y los ensayos toxicológicos que se han llevado a cabo con las mismas. Los resultados publicados por diversos autores presentan resultados contradictorios en muchos casos (Sharma y col., 2010; Baek y col., 2012), haciendo necesaria una evaluación toxicológica exhaustiva y consideración individualizada de cada una. En el caso de extractos de migración obtenidos a partir de materiales nanocompuestos, los estudios son mucho más escasos (Avella y col., 2005; Simon y col., 2008).

Debido al auge que está presentando el uso de arcillas y nanocompuestos poliméricos de arcilla, sería necesaria hacer una revisión bibliográfica, caso por caso, con el fin de ir recopilando la mayor información posible y poder determinar el estado del arte de cada material con el fin de encaminar y completar una adecuada evaluación toxicológica y ser conscientes de la información sobre seguridad que hay hasta el momento.

La Autoridad Europea de Seguridad Alimentaria (EFSA) es la piedra angular de la Unión Europea (UE) centrada en la evaluación de riesgos en relación con la alimentación y la seguridad de los piensos. En estrecha colaboración con las autoridades nacionales y en consulta abierta con grupos de interés, la EFSA proporciona asesoramiento científico independiente y comunicación clara sobre los riesgos existentes y emergentes. La EFSA ha desarrollado un enfoque práctico para evaluar los riesgos potenciales derivados desde las aplicaciones de la nanociencia y la nanotecnología en la cadena alimentaria humana y animal (EFSA, 2011). En este

aspecto, para llevar a cabo una rigurosa evaluación de las arcillas, como nanoarcillas, se tienen en cuenta los pasos propuestos por esta autoridad, con el fin de ser comercializadas en un futuro cercano en Europa.

En su informe, la EFSA proporciona orientación sobre: (I) los requisitos de caracterización físico-química de nanomateriales artificiales utilizados por ejemplo como aditivos alimentarios, enzimas, aromatizantes, materiales en contacto con alimentos, alimentos nuevos, aditivos para piensos y plaguicidas y; (II) las pruebas para identificar y caracterizar los peligros derivados de las propiedades de los materiales en evaluación que, en general, incluye, información sobe genotoxicidad *in vitro*, absorción, distribución, metabolismo y excreción, además de ensayos de toxicidad oral de dosis repetida durante 90 días en roedores. Esta guía indica todas las incertidumbres que deben ser consideradas para llevar a cabo una correcta y completa evaluación de riesgo (EFSA, 2011a).

Además, la EFSA ha publicado recientemente, "*Acontecimientos recientes en la evaluación del riesgo de sustancias químicas en alimentos y su potencial impacto en la evaluación de la seguridad de sustancias utilizadas en los materiales en contacto con alimentos*", un boceto-guía con estructura similar a la anteriormente mencionada, destacando que una evaluación genotóxica es requerida en cualquier caso (EFSA, 2015). Éste último documento completaría en parte a la información proporcionada por el primero.

Muchos de estos aspectos toxicológicos de las nanoarcillas han sido abordado por numerosos autores. A continuación se expondrán los mecanismos de toxicidad que hasta el momento se han descrito sobre los materiales que nos competen, esbozando resultados disponibles más relevantes.

5.1. ENSAYOS TOXICOLÓGICOS *IN VITRO* Y MECANISMOS DE TOXICIDAD

Los métodos *in vitro* proporcionan un medio efectivo y rápido para la selección (*screening*) y clasificación de compuestos, además de ser ética y moralmente más correctos que los ensayos *in vivo*. Entre otras ventajas destacan que el uso de animales es considerablemente menor, e incluso nulo en muchas ocasiones, se utiliza material muy homogéneo obtenido con técnicas estandarizadas, posibilitan el uso de material de

origen humano, lo que se puede simplificar por extrapolación, son más fácilmente objetivables y cuantificables que los ensayos *in vivo* y los resultados presentan mayor reproducibilidad. A todo ello se le une un menor coste, mayor rapidez e instalaciones menos complejas (Eisenbrand y col., 2002).

Las evaluaciones toxicológicas *in vitro* evaluaciones toxicológicas son útiles para la obtención de información acerca del mecanismo de toxicidad de los materiales de estudio. Las células responden rápidamente al estrés que genera el tóxico mediante la alteración, por ejemplo, de diferentes biomarcadores basales, tales como la generación de especies reactivas de oxígeno (ERO) y contenido de glutatión (GSH), o alteraciones en varios orgánulos, como las mitocondrias y lisosomas. Teniendo esto en cuenta, una cuestión importante de resolver es cómo los materiales estudiados, en este caso, organoarcillas y nanocompuestos que las contengan, son capaces de alterar estos parámetros y cómo estas alteraciones pueden afectar a la tasas metabólicas, crecimiento celular y la transcripción génica (Eisenbrand et al., 2002).

La interacción, influencia y potencial de toxicidad de los materiales con las proteínas y las células son un enfoque esencial para evaluar y entender la compatibilidad de los materiales frente a la toxicidad (Jones y Grainger, 2009). Las reacciones de células de interés como material de absorción celular, las perturbaciones integridad de la membrana, o la alteración en la viabilidad de orgánulos vitales. Por lo tanto, los puntos finales tales como la generación de ERO y otros parámetros relacionados con la respuesta al estrés celular (GSH, la actividad de la superóxido dismutasa (SOD), la peroxidación lipídica (LPO), etc.), daño en el ADN, la respuesta de la inflamación y la necrosis y apoptosis proporcionan una valiosa información para la comprensión de la respuesta tóxica.

Entre los indicadores de toxicidad que se pueden determinar con los métodos *in vitro*, destacan que a continuación se exponen.

a) Citotoxicidad basal

Los cultivos de células juegan un papel esencial en la evaluación toxicológica de los minerales de arcilla, ya que proporcionan los sistemas de alto rendimiento para la detección rápida y rentable de los peligros. Seleccionadas las líneas celulares de trabajo, teniendo un gran interés las líneas de órganos diana del aparato digestivo, los ensayos de citotoxicidad son los llevados a cabo en primera instancia para valorar los efectos

adversos o interferencias con estructuras y/o propiedades esenciales para la supervivencia, proliferación y/o función celular una vez que se les ha expuesto el compuesto objeto de estudio.

Diversos grupos han evaluado mediante diferentes biomarcadores de citotoxicidad la alteración funcional producida por exposición de diferentes líneas celulares a dichas arcillas organomodificadas.

Son diferentes los métodos que podemos encontrar para evaluar el descenso de viabilidad tras exposición de las arcillas, así como extractos de migración de materiales nanocompuestos. Entre ellos destacan, la reducción de Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) y sal de tetrazolio MTS (MTS) (Baltrop y col., 1991), comprobando la funcionalidad mitocondrial tras la exposición al compuesto; liberación de la enzima Lactato Deshidrogenasa (LDH) para evaluar el daño en la membrana celular; el ensayo Azul Trypan (Binderup y col., 2002) para evaluar la proliferación celular; el ensayo de Rojo Neutro (RN) (Borenfreud y Puerner., 1984) que constata el daño lisosomal y la valoración del contenido de proteinas (Bradford, 1976), con el fin de evaluar la proliferación celular.

La Organización Mundial de la Salud (OMS) revisó los efectos en sistemas *in vitro* de bentonita, caolín y otros minerales de arcilla (OMS, 2005). Sin embargo, las obras mencionadas fechan del período 1969-1996, no resultando ser una información actual y extrapolable a la realidad, haciéndose de tal forma necesaria una profunda revisión de los datos disponibles hasta día de hoy.

Estudios más actuales han demostrado diferentes comportamientos de líneas celulares tras la exposición de la Mt o Cloisite®Na^+ (CNa^+). Por ejemplo, Sharma y col., (2010) observaron un efecto no citotóxico en la línea de adenocarcinoma de colon Caco-2 expuesta a la arcilla en las condiciones ensayadas, sin embargo, Li y col. (2010) y Baek y col., (2012), obtuvieron un descenso de viabilidad tras la exposición de la misma en la línea celular de ovario de hámster chino (CHO) y en células intestinales humanas, respectivamente.

Respecto a otras arcillas modificadas disponibles comercialmente, los resultados obtenidos también fueron muy diversos, presentándose de forma general efectos tóxicos tras la exposición a las arcillas. En el caso de Cloisite®30B (C30B) y Cloisite®93A

(C93A)) se observaron efectos citotóxicos tras su exposición en Caco-2 y la línea de hepatoma humano HepG2 (Sharma y col., 2010; Lordan y col., 2011). Por otro lado, Liu y col. (2011) también observaron un descenso de la viabilidad celular y un aumento en la liberación de lactato deshidrogenasa (LDH) en fibroblastos embrionarios de ratón (NIH 3T3) y células de riñón embrionario 293 (293 HEK) tras la exposición a una Mt modificada con oligo (estireno-co-acrilonitrilo).

En definitiva, cada arcilla, puede presentar un perfil toxicológico diferente teniendo en cuenta las líneas celulares expuestas, tiempos ensayados y concentraciones de exposición, obteniendo de tal forma una amplia diversidad en los resultados, haciéndose necesaria una evaluación caso por caso.

Respecto los ensayos de citotocidad llevados a cabo con extractos de migración de materiales nanocompuestos poliméricos de arcilla son muy escasos hasta el momento, siendo necesaria una exhaustiva investigación de los mismos antes de la comercialización del envase. Zia y col. (2011), obtuvieron una mejor adhesión de células en envases con arcillas sin modificar que con arcillas modificadas, viendo efectos adversos ante la presencia de modificadores.

b) Estrés oxidativo

La expresión / generación de respuestas de estrés y las alteraciones bioquímicas posteriores puede ser potenciales marcadores para la toxicidad inducida por el compuesto (Eisenbrand et al., 2002). Existe una considerable evidencia que indica que muchas de las respuestas de estrés se producen antes de que cualquier citotoxicidad medible; por lo tanto, también se recomienda la vigilancia de las vías de estrés a nivel subtóxicas. La producción de formas reactivas de oxígeno es tóxica para los organismos y un exceso de las mismas da lugar a lo que se conoce como estrés oxidativo (**Fig. 7**).

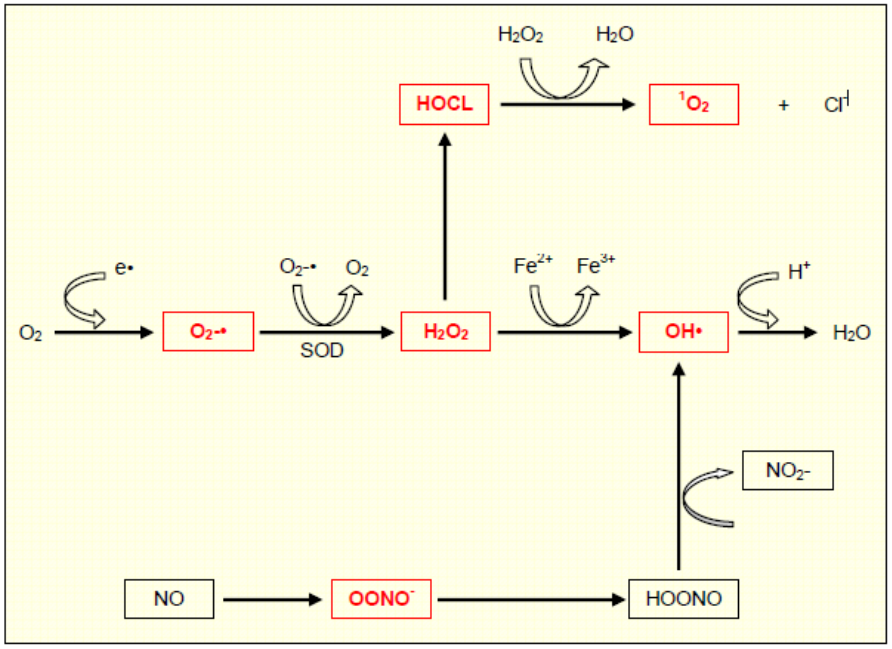

Figura 7. Principales especies reactivas de oxígeno (tomada de Guzmán-Guillén, 2015).

La estructura electrónica del oxígeno hace que sea susceptible de reducciones parciales, dando lugar a especies reactivas de oxígeno (ERO). Las ERO se producen normalmente en las células como producto del metabolismo celular. Si un agente estresante induce un aumento en la producción de ERO, este equilibrio puede romperse causándose daños en las estructuras celulares, tales como disfunción de la membrana, peroxidación lipídica (LPO), lo que significa un mayor nivel de malondialdehído (MDA) en la membrana celular, daño de ADN y una inactivación drástica de las proteínas. La concentración de ERO está controlada por acción de la barrera de defensa antioxidante enzimática. Ejemplos de estas enzimas son la superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) y glutatión reductasa (GR) (Puerto et al., 2009, 2010) (**Fig. 8**).

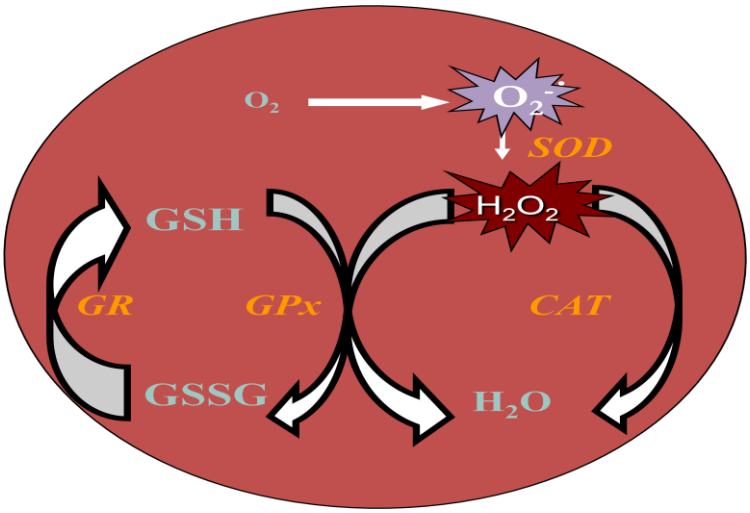

Figura 8. Principal barrera antioxidante enzimática del organismo (Tomada de Guzmán-Guillén, 2015).

Las mediciones de actividad de este grupo de enzimas antioxidantes pueden proporcionar un marcador de estrés oxidativo, lo que se puede completar con el estudio de otros parámetros, tales como el GSH, MDA y los niveles de carbonilo (Puerto y col., 2014). Si no se recupera el equilibrio se producirán disfunciones que pueden desembocar en la muerte celular.

Del mismo modo que para los ensayos de citotoxicidad basal, los resultados que encontramos en bibliografía acerca de generación de ERO e inhibición de la síntesis de GSH suelen ser contradictorios y no concluyentes. Sharma y col. (2010) no encontraron una respuesta en la que se generara ERO tras la exposición de CNa+ y C30B a Caco-2, sin embargo, otros autores confirmaron la producción de estrés oxidativo con CNa^{+} expuesta a HepG2 y células intestinales humanas (Lordan y col., 2011; Baek y col., 2012). Además, tras la exposición de diferentes líneas celulares a otras arcillas comerciales, como C93A en HepG2, también se observó un incremento en la generación de ERO (Lordan y col., 2011).

Poco se sabe acerca de la producción de estrés oxidativo por extractos de migración de materiales nanocompuestos derivados de arcilla destinados al envasado al alimento, siendo necesaria dicha información previa comercialización de estos productos. Podemos encontrar estudios acerca de otros materiales nanocompuestos poliméricos de arcilla destinados a otros usos médicos, observándose una ausencia de estrés según los biomarcadores evaluados (MDA, SOD, GSH, etc) (Kevadiya y col., 2013,2014).

c) Genotoxicidad/Mutagenicidad

Entre los efectos que podemos encontrar por exposición de concentraciones subcitotóxicas de productos químicos se encuentra la interacción directa con el ADN, traduciéndose dicha interacción en diversos tipos de daño, incluyendo lesiones promutagénicas, que provocan la activación de protooncogenes y la inactivación de genes supresores de tumores (Kensler y col., 2003). Las lesiones genéticas no sólo son un reflejo de acontecimientos inducidos por el compuesto, sino también indicadores de inestabilidades genéticas causadas por deficiencias de la reparación del ADN (Eisenbrand y col., 2002).

Debido a la variedad de mecanismos que conducen al daño del ADN inducido por xenobióticos y el abanico de acontecimientos mutágenos que pueden ocurrir como resultado, se requiere una batería de ensayos para el establecimiento del potencial genotóxico de una sustancia que se encuentre bajo investigación (Doak y col., 2012). En la actualidad, los ensayos de mutagenicidad/genotoxicidad *in vitro* se incluyen entre el conjunto básico de pruebas de toxicidad para la evaluación de la seguridad requerida por la legislación europea para autorizar diferentes sustancias que se pretendan emplear en la industria alimentaria (EFSA, 2011b). Los ensayos de genotoxicidad de uso más frecuente son el el ensayo de micronúcleos (MN), el Test de Ames y el ensayo cometa, (**Fig.9**).

De acuerdo con la directriz de la OCDE 487 (OCDE, 2014), el ensayo *in vitro* de MN es una prueba que determina rápidamente la frecuencia de daño cromosómico bruto inducida por un agente de estudio. Esta técnica, ha ganado popularidad frente al ensayo de aberraciones cromosómicas, ya que es sustancialmente es más rápida de realizar, más fácil de analizar y detecta fácilmente compuestos aneugénicos y clastogénicos (que el ensayo de aberración cromosómica no puede hacerlo a menos que se modifique específicamente). Por lo tanto, la MN actualmente se recomienda y exige como uno de los sistemas de ensayo *in vitro* para caracterizar la genotoxicidad de nuevas sustancias (EFSA, 2011b; Kirkland y col., 2011; EFSA, 2015).

Otro ensayo realizado para evaluar diferentes alteraciones que podrían ocurrir en el material genético es el Test de Ames. Dicha prueba, de acuerdo con la directriz de la OCDE para los ensayos de productos químicos 471 (OCDE, 1997), es el ensayo de mutación inversa bacteriana utilizado para determinar la mutagenicidad de sustancias

exógenas. El ensayo identifica la capacidad de compuestos de revertir mutaciones puntuales los genes de la biosíntesis de histidina o triptófano, en *Salmonella typhimurium* o *Escherichia coli*, respectivamente, restaurándose la capacidad de las bacterias para generar estos aminoácidos esenciales. Por lo general, una combinación de 5 cepas de S. typhimurium o 4 cepas de *S. typhimurium*, además de 1 o 2 cepas de *E. coli* se exigen para detectar mutaciones por sustitución de bases o desplazamiento en el marco de lectura. La facilidad y la rentabilidad de este ensayo hacen que sea ampliamente utilizado en el análisis de seguridad de xenobióticos. Es una prueba esencial dentro de la batería actual de ensayos requeridos para la evaluación de la genotoxicidad (EFSA, 2011b; Doak y col., 2012; EFSA, 2015).

El ensayo cometa (electroforesis en gel de una sola célula) es un método simple para medir roturas de la cadena de ADN en células eucariotas (Collins, 2004). Este ensayo presenta una serie de ventajas en comparación con otras pruebas de genotoxicidad. Además de la capacidad de este ensayo para identificar el daño del ADN a nivel de una sola célula, entre otras de las ventajas significativas se incluyen: su sensibilidad para la detección de bajos niveles de daño en el ADN, requerimiento de un pequeño número de células por muestra, su fácil de aplicación, y, bajo costo y tiempo necesario para la realización del ensayo. Además, este ensayo es flexible porque puede ser usadao para evaluar diversos tipos de daño del ADN, como el oxidativo, y es fácilmente modificable para la adaptación a una variedad de requerimientos experimentales (Tice y col., 2000). Por lo tanto, no es de extrañar que el ensayo cometa haya y esté siendo utilizado en muchos estudios para investigar el daño y reparación del ADN en una amplia gama de células tumorales con una extensa variedad de agentes que dañan el ADN (Liao ey col., 2009).

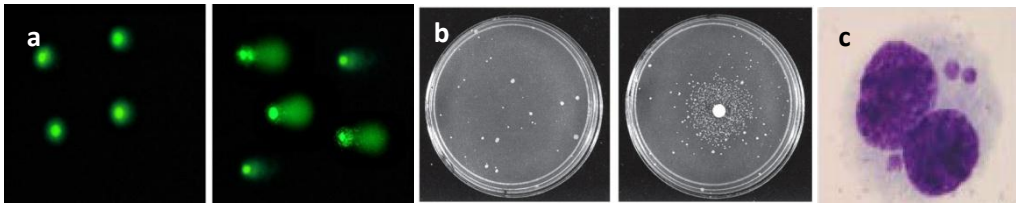

Figura 9. Visualización del resultado del Ensayo Cometa (a), Test de Ames (b) y Micronúcleos (c).

En este sentido, la evaluación genotoxicidad de las arcillas y nanocompuestos derivados utilizados o destinados para la industria alimentaria es de gran interés y está exigida en cualquier caso, tal y como ha publicado la EFSA recientemente (EFSA, 2015).

Respecto a los ensayos de genotoxicidad llevados a cabo con arcillas, destacar que siguen presentándose datos no concluyentes. Li y col. (2010) observaron la ausencia de genotoxicidad mediante los tres ensayos descritos de láminas de silicatos, mientras que Sharma y col. (2010) obtuvieron una respuesta genotóxica y mutagénica positiva para C30B. Por otro lado, hasta el momento, bajo nuestro conocimiento, los estudios de genotoxicidad realizados sobre materiales nanocompuestos son inexistentes.

d) Marcadores inflamatorios

La inflamación es un intento de protección por el organismo para eliminar los estímulos nocivos y para iniciar el proceso de curación. Durante este proceso, algunos biomarcadores pueden ser liberados, determinándose si la exposición a una sustancia en particular puede conducir a una respuesta del sistema inmune. Entre los biomarcadores que se analizan con mayor frecuencia en relación con el proceso de inflamación están la liberación de citoquinas, principalmente la interleucina-6-factor α (IL-6) y la necrosis tumoral (TNF-α) (Lopresti y col., 2014).

Actualmente, podemos mencionar por ejemplo la evaluación realizada por Elmore (2003), que publicó que diferentes arcillas, incluída la Mt, inducían citotoxicidad en diferentes líneas celulares de macrófagos y que se presenciaba una respuesta hemolítica en líneas celulares sanguíneas de diferentes especies.

e) Muerte celular

Diferentes marcadores pueden ser utilizados para determinar el mecanismo de la muerte celular después de la exposición a una sustancia tóxica. La citometría de flujo y anexina V se utilizan para determinar cuantitativamente el porcentaje de células dentro de una población que están experimentando apoptosis de forma activa, ya que se unen los fosfolípidos de membrana externalizados (Napierska y col., 2009). Por otra parte, otro biomarcador importante es la actividad de caspasas, principalmente la actividad de la caspasa 3, que es considerada la más importante de las caspasas efectoras y su

activación se utiliza a menudo como un indicador de un evento apoptótico (Lordan y col., 2011).

Lordan y col. (2011) observó una ausencia en el incremento de actividad capasas tras exposición de CNa^+ en una línea celular hepática diana; sin embargo, Janer y col. (2014) sí obtuvieron un incremento en diferentes cultivos celulares tras la exposición de Mt, a tiempos tanto cortos como más amplios de exposición. Por otro lado, Geh y col. (2006), también observaron como respuesta de muerte celular necrosis y apoptosis tras exposición de bentonita.

5.2. **ENSAYOS TOXICOLÓGICOS *IN VIVO***

Estos ensayos incluyen la experimentación hecha dentro o en el tejido vivo de un organismo vivo. Los ensayos *in vivo* enriquecen la información preliminar obtenida de los ensayos *in vitro*. Éstos dan una información más completa de lo que ocurre en el organismo cuando se encuentra expuesto a cualquier xenobiótico objeto de estudio, proporcionando datos extrapolables al ser humano y pudiendo detectar efectos secundarios. Son métodos validados formalmente y son los que comúnmente se llevan a cabo para los ensayos clínicos y registro de nuevos fármacos, además de otras nuevas sustancias.

Los ensayos *in vivo* llevados a cabo con arcillas y materiales nanocompuestos son más escasos que los publicados *in vitro*, y la mayoría se centran en cortos periodos de exposición. Varios autoreshan llevado a cabo estudios de toxicidad oral evaluando diferentes parámetros tras exponer a roedores a concentraciones mucho más elevadas que las usadas *in vitro*. En el caso de Li y col. (2010) y Baek y col. (2012) no observaron ninguna anomalía ni pérdida de peso en los animales durante el periodo de tratamiento con láminas de nanosilicato. Sin embargo, otros autores han mostrado como nontronitas y hectoritas, otro tipo de arcillas, han dado lugar a la generación de LPO en el cerebro de roedores expuestos a altas concentraciones (Kibanova y col., 2009).

Por otro lado, la EFSA llevó a cabo la evaluación de la bentonita como aditivo de alimentación, no encontrando resultados de genotoxicidad mediante el ensayo de aberraciones cromosómicas *in vivo* (EFSA, 2013). Además, la Agencia Internacional de Investigación sobre el Cáncer (IARC) no obtuvo ningún dato sobre la carcinogenicidad de la Mt o caolinita (IARC, 1997).

Respecto a la absorción y acumulación en el organismo de este tipo de compuestos poco se sabe, siendo muy limitados los estudios encontrados en bibliografía, requiriéndose una profunda revisión debido a la antigüedad de muchos de los artículos disponibles. Destacar algunos resultados publicados que constatan la acumulación progresiva en orina y en determinados órganos (riñón>hígado>corazón>cerebro) de restos de arcillas destinadas a diferentes usos en el ámbito de la medicina (Mascolo y col., 1999; 2004). Sin embargo, en el informe mencionado de la EFSA, ni bentonita ni sepiolita, comparadas con otras arcillas, presentan un grado de absorción medible (EFSA, 2013).

En cuanto a la evaluación toxicológica *in vivo* de materiales nanocompuestos de arcillas sólo hemos encontrado en bibliografía un único trabajo, donde se ve una mayor compatibilidad *in vivo* de un polímero reforzado con Mt comparado con el polímero puro (Hsu y col., 2012).

5.3.ENSAYOS TOXICOLÓGICOS A NIVEL MOLECULAR

La evaluación de efectos a nivel molecular nos da una información a nivel génico y proteico de los efectos desencadenados tras la exposición a un xenobiótico. En este sentido los datos obtenidos completarían el perfil toxicológico de una sustancia, ya que los ensayos llevados a cabo para determinar estos parámetros pueden realizarse tanto *in vitro* como *in vivo*. Los ensayos más destacados a nivel molecular son la Reacción en Cadena Polimerasa Cuantitativa a Tiempo Real (qPCR), que evalúa la expresión génica, y, el Western Blot, destinado a la evaluación de la abundancia proteica.

La invención de la reacción en cadena de la polimerasa (PCR) por K. Mullis y sus colaboradores en 1985 ha revolucionado la biología y la medicina molecular (Saiki y col., 1985). La reacción en cadena de la polimerasa es una técnica *in vitro* utilizada para amplificar enzimáticamente una región determinada de ADN situada entre dos regiones de ADN cuya secuencia se conoce. Mientras que antes solo podían obtenerse cantidades mínimas de un gen específico, ahora incluso un único ejemplar de un gen puede amplificarse con la PCR hasta un millón de ejemplares en poco tiempo. Las técnicas de PCR se han hecho indispensables para muchos procedimientos comunes, como la clonación de fragmentos específicos de ADN, la detección e identificación de genes para diagnóstico y medicina legal, y en la investigación de modelos de expresión

de los genes, siendo este último el aspecto que más nos interesa. Más recientemente, la PCR ha permitido la investigación de nuevos campos, como el control de la autenticidad de los alimentos, la presencia de ADN modificado genéticamente y la contaminación microbiológica (Somma y Querci, 2007)**.**

En el caso de la qPCR, se mide la cantidad de ADN después de cada ciclo por el uso de marcadores fluorescentes que se incorporan en la reacción final o bien en la mezcla del gen seleccionado (sonda+gen). El aumento de la señal fluorescente es directamente proporcional al número de moléculas del gen (amplicones) generados en la fase exponencial de la de reacción. Se usan indicadores fluorescentes que se incluyen en el ADN de doble cadena a colorantes de unión, moléculas de colorante unidas a los cebadores de PCR o sondas que son incorporadas en el producto durante la amplificación. Se mide el cambio en la fluorescencia durante el transcurso de la reacción por un instrumento que combina el ciclo térmico con capacidad de escaneado. El instrumento de qPCR genera una trama de amplificación que representa la acumulación de producto durante la duración de toda la reacción de PCR, finalmente se compara con la expresión de un estándar conocido normalizándose de tal forma la cantidad expresada del gen seleccionado. Los datos obtenidos mostrarán una baja o sobreexpresión con respecto al grupo control, determinando el tipo de afectación producido por la sustancia objeto de estudio (Invitrogen, Life Technologies).

Por otro lado, el Western blot se utiliza a menudo en la investigación para separar e identificar las proteínas. Esta técnica se centra en la separación de proteínas en base al peso molecular de cada una a través de electroforesis en gel. Estos resultados son transferidos a una membrana donde aparece una banda para cada proteína. La membrana se incuba a continuación con anticuerpos específicos para la proteína de interés, uniéndose a ella y dando lugar a la banda de señal. El espesor de la banda corresponde a la cantidad de proteína presente; por lo tanto, haciendo un estándar puede indicar la cantidad de proteína presente (Mahmood y Yang, 2012).

En literatura no existen hasta el momento estudios que evalúen la influencia de las arcillas destinadas a la industria alimentaria en la modulación de la expresión génica y abundancia proteica tras su exposición.

6. COMERCIALIZACIÓN Y ASPECTOS REGULATORIOS

Como hemos estado viendo a lo largo de esta introducción, no sólo es importante la optimización de procesos y la creación de productos nanocompuestos poliméricos de arcilla destinados al mercado, sino que también hay otras dos cuestiones que hasta el momento han recibido poca atención: la migración y la toxicidad de estos nuevos materiales. De esta forma, la evaluación de organoarcillas debe centrarse no solo en la arcilla modificada incorporada ya al polímero, sino también, en la propia arcilla antes y después de la modificación e incorporación.

Hasta la fecha, no existe una evidencia científica rotunda que sugiera motivo de preocupación por el uso de arcillas modificadas; sin embargo, es lógico que los nuevos alimentos y materiales de envasado deben ser minuciosamente evaluados con el fin de cumplir con los requisitos de la EFSA y otros organismos reguladores, siendo probable que la investigación sobre propiedades de migración y la toxicidad de polímeros nanocompuestos usados en la industria alimentaria como materiales en contacto con alimentos se amplíe en un futuro muy cercano (Plackett y Siró, 2012).

Si bien es cierto, que hasta el momento no se han comercializado en Europa, productos nanocompuestos de arcilla destinados al envasado de alimentos, la EFSA, si propone diferentes guías, ya mencionadas en el apartado 5, para la evaluación de nuevos materiales destinados al contacto de alimentos (EFSA, 2011a, 2011b, 2015).Para ello, sería necesaria realizar una exhaustiva evaluación en la actualidad, con el fin de que en un futuro temprano se puedan comercializar estos envases mejorados, y sobre todo, seguros.

Además, es muy importante destacar el Reglamento UE Nº 10/2011 de la Comisión de 14 de enero de 2011 sobre materiales y objetos plásticos destinados a entrar en contacto con alimentos y la Directiva del Consejo del 18 de octubre de 1982, por la que se establecen las normas básicas necesarias para evaluar la migración de los constituyentes de materiales y objetos plásticos destinados a entrar en contacto con productos alimenticios. En ambos se recogen los aspectos relacionados con los materiales plásticos y objetos destinados a entrar en contacto con los alimentos (en nuestro caso, materiales nanocompuestos poliméricos de arcilla) y los productos alimenticios, así como los simuladores utilizados en los ensayos de migración,

estableciendo que la evaluación del riesgo debe cubrir la migración potencial en las peores condiciones previsibles de uso y toxicidad.

Por otra parte, el Reglamento UE Nº 10/2011 de la Comisión también establece un límite de migración global de 10 mg / dm^2, basándose en que los materiales plásticos y artículos derivados no deberán ceder sus componentes a los alimentos simuladores en cantidades superiores a 10 mg de los componentes totales liberados por dm^2 de superficie en contacto con el alimento.

Como curiosidad, comentar que en otros países ajenos a la UE, como es el caso de EE.UU., podemos encontrar materiales ya comercializados y aprobados por la Admistración de Drogas y Alimentos (U.S. Food and Drug Administration, FDA), Autoridad Americana de Alimentación, homóloga a la EFSA en Europa. Por ejemplo, Bayer Polymers (Pittsburgh, PA, EE.UU) ha introducido Durethan®, una poliamida que contiene silicato en capas como nanorrelleno para proveer mejores propiedades contra el gas y la humedad al polímero, y así proporcionar una mayor vida útil a los productos alimenticios (Anon, 2009). Nanocor (Arlington Heights, IL, EE.UU.) anuncia la oferta de nanocristales que pueden ser usados para mejorar las propiedades de barrera de plásticos de botellas de cerveza y mejorar así la vida útil de la bebida. Además, el ejército de EE.UU. ha llevado a cabo una investigación acerca de la producción de bolsas de comidas preparadas listas para consumir a base de polietileno de baja densidad con un refuerzo de un 7,5% de lámina de silicatos, más específicamente Mt, como nanorelleno. Por la incorporación de estás láminas de silicato se han conseguido mejoras térmicas, mecánicas y de barrera muy significativas comparadas con el material de partida (National Nanotechnology Initiative, 2009). Por otro lado, Honeywell ofrece seis resinas con diferentes grados de rellenos basados en nanoarcillas-nylon, bajo el nombre comercial Aegis®. Estos productos han sido utilizados por Anchor Brewing Company (San Francisco) para botellas de cervezas y films especiales. Un ejemplo de estos materiales es Nanocor Nano-PA-6,conocido por ser el primer plástico comercial nanocompuesto usado como empaquetado flexible, así como revestimiento para cartón con fin de aumentar la barrera contra la humedad y el oxígeno, por ejemplo para tetrabricks de leche y zumos. Además se ha visto que proporciona una mejor rigidez y resistencia al calor. Por otro lado, otros nanocompuestos de resinas de Nanocor se han utilizado para botellas multilaminadas de polietileno (PET) y en botellas que alberguen alimentos sensibles al dióxido de carbono y al oxígeno. En diciembre de 2009, la FDA

aprobó la comercialización de el producto Nanocor-Mt, tratado con sales de amonio cuaternario, destinado a incrementar las propiedades barrera de botellas de PET (Plackett y Siró, 2012).

Siempre que sea posible, teniendo en cuenta como fin último la seguridad de los consumidores, la producción de polímeros nanocompuestos en áreas especializadas podría tener ventajas significativas en términos de manejo y seguridad, especialmente teniendo en cuenta los materiales para envases de alimentos y bebidas.

7. OTROS USOS Y VÍAS DE EXPOSICIÓN

A lo largo de la historia las arcillas han sido utilizadas por el ser humano con fines terapéuticos. Los minerales de arcilla pueden ser beneficiosos para la salud humana, sirviendo como principios activos o excipientes en forma de preparados farmacéuticos, en los balnearios y en la medicina terapéutica de belleza (Carretero y col., 2006).

El uso de minerales de arcilla en las formulaciones farmacéuticas ha sido descrito por muchos autores, destacando el uso de caolinita, talco, paligorskita, y esmectitas, como principios activos o excipientes de las mismas (Viseras y López-Galindo, 1999; López-Galindo y Viseras, 2000; Carretero, 2002). Las propiedades fundamentales por las que los minerales de arcilla se utilizan en la industria farmacéutica son las comentadas a lo largo de secciones anteriores: el alta área específica y capacidad de absorción, contar con características reológicas favorables, la inercia química, baja o nula toxicidad para el paciente (aspecto que habría que corroborar), y bajo precio (Carretero y col., 2006).

Las arcillas pueden ser administradas a los pacientes como protectores gastrointestinales, antiácidos y/o antidiarreicos, bien por vía oral en forma de píldoras, polvos, suspensiones, y emulsiones, o bien, por vía tópica usándose como protectores dermatológicos o por razones de estéticas.

Además, la caolinita, el talco, la paligorskita, y las esmectitas también se utilizan como excipientes en cosmética y preparaciones farmacéuticas, funcionando como: lubricantes para facilitar la fabricación de pastillas; agentes para ayudar a la desintegración a través de su capacidad de hincharse en presencia de agua o por medio

de la dispersión de fibras, promoviendo la liberación de la droga cuando llega al estómago; y, como emulsionantes, geles polares y agentes espesantes por sus características coloidales, evitando la segregación de los componentes de la formulación farmacéutica y la formación de un sedimento que precipite y no se distribuya (Carretero y col., 2006).

Por otro lado, Carretero y col. (2006) también describieron que las arcillas pueden utilizarse también en spas y tratamientos de belleza, como es el caso de las caolinitas y esmectitas.

Las principales propiedades de los minerales de arcilla que determinan su utilidad en el spa y medicina estética, son: a) la suavidad y el pequeño tamaño de la partícula, ideal para la aplicación del lodo o la mascarilla facial o corporal; b) las propiedades reológicas adecuadas para la formación de una viscosa y consistente pasta, y buenas propiedades plásticas para una fácil aplicación y adhesión a la piel durante el tratamiento; c) la similitud en el pH al de la piel con el fin de evitar la irritación u otra alteración dermatológica; d) gran capacidad de absorción(las arcillas pueden eliminar el exceso de grasa y las sustancias tóxicas de la piel, siendo muy eficaces contra las enfermedades dermatológicas tales como forúnculos, acné, úlceras, abscesos y seborrea); e) alta capacidad de intercambio iónico, permitiendo un intercambio de nutrientes (Ca^{+} ó Na^{+}) mientras la arcilla está en contacto con la piel; f) alta capacidad de retención de calor. Como el calor es también un agente terapéutico, las arcillas se aplican en caliente para tratar inflamaciones reumáticas, traumatismos deportivos crónicos, y problemas dermatológicos.

A parte de las dos vías de exposición ya nombradas, la oral y la dérmica, la vía de entrada de minerales de arcillas al organismo más común es la vía inhalatoria (Zhao y Castranova, 2011; Silvestre y col., 2011). No obstante, en la actualidad existe escasa información sobre la exposición ocupacional a los minerales de arcilla en las minas, plantas de procesamiento, y las industrias. Sin embargo, se saben que el altos niveles de minerales de arcilla o la exposición inadecuada a estos en el medio ambiente y los seres humanos pueden dar lugar a efectos indeseables (Zhu y Njuguna, 2014).

Los polvos minerales causan daños por inhalación, y rara vez por ingestión o penetración en la piel. En los pulmones, los minerales pueden producir diversas patologías como cáncer de pulmón o mesotelial y neumoconiosis. Las patologías

provocadas por minerales puede ser determinada mediante estudios epidemiológicos, basados en la evaluación de la relación que hay entre la exposición humana a una sustancia peligrosa y los efectos potenciales para la salud que se pueden dar tras esa exposición; además puede evaluarse mediante estudios *in vivo* e *in vitro*, estudiando los efectos de la exposición a polvos minerales en modelos animales o bien determinando la actividad biológica de un mineral en líneas celulares específicas, respectivamente (Guthrie, 1992).

Aunque los datos son antiguos, hasta el momento, el efecto nocivo causado por inhalación de la caolinita, está principalmente relacionado con la presencia de diversos minerales en las rocas, entre los que se encuentra el cuarzo. Trabajadores expuestos al polvo de la caolinita han desarrollado en muchos casos neumoconiosis; sin embargo no hay un gran número de datos relacionados con el cáncer de pulmón (Ross y col., 1993).

Los estudios epidemiológicos llevados a cabo con trabajadores expuestos a sepiolita no mostraron datos que supusiesen riesgo de padecer enfermedad pulmonar, pudiendo concluir que la exposición a este tipo de minerales no supone ningún riesgo (Mc Connochie y col., 1993; Ross y col., 1993; Santaren y Alvarez, 1994).

Respecto al grupo de las esmectitas, en bibliografía científica hay pocos estudios epidemiológicos disponibles de enfermedades respiratorias causadas por la exposición a ellas. Algunos estudios sugieren que determinadas muestras pueden provocar una respuesta fibrogénica dependiente de la dosis ante altos niveles de exposición. Generalmente, sin embargo, hay una exposición concomitante a otros minerales (por ejemplo, sílice y anfíboles), complicándose la interpretación de los datos obtenidos. Así, los depósitos de bentonita generalmente contienen otros minerales, incluyendo cuarzo de grano muy fino y sílice. En el caso de Wyoming (EE.UU.) el contenido de bentonitas en sílice y cuarzo oscila entre un 0 a 24% (Ross y col., 1993).

En conclusión, para todo tipo de aplicaciones sería necesaria una evaluación caso por caso, teniendo en cuenta tipo de arcilla, tiempo y vía de exposición, y dosis empleadas como factores más importantes para una correcta evaluación de riesgo.

8. ACEITES ESENCIALES, OTRA ALTERNATIVA PARA EL ENVASADO ALIMENTARIO

La industria alimentaria, como hemos visto en el caso de materiales nanocompuestos, con idea de suplir carencias presentes en la conservación de alimentos, han hecho necesaria la aparición de un nuevos tipos de envases que permitan cubrir tanto las necesidades de los consumidores, enfocadas a alimentos más sanos y naturales, como las derivadas de la globalización de la alimentación, que necesita alimentos que permanezcan con sus cualidades inalteradas durante períodos de tiempo más prolongados.

Los materiales nanocompuestos poliméricos de arcilla son una alternativa real en diversas zonas del mundo, que persigue el aumento de la vida útil del alimento centrándose en las propiedades tecnológicas del envasado en sí, dando lugar a las mejoras descritas, pero no ejerce ningún efecto directo sobre el propio alimento.

Sin embargo, no es la única opción que se baraja dentro del área del envasado, valorándose y desarrollándose una perspectiva ecológica y sostenible, compartida por las empresas de la industria alimentaria, el envasado activo (Tharanathan, 2003). Además, la presión ejercida por parte de los consumidores, siendo cada vez más conscientes de los problemas derivados del uso de aditivos sintéticos en los alimentos, unida a la necesidad de gestionar los subproductos de las industrias y buscar fuentes renovables para la creación de polímeros, hace que empiecen a desarrollarse envases con compuestos naturales (Llana-Ruíz Cabello, 2014).

El envasado activo permite que los alimentos puedan estar en una atmósfera natural, donde constituyentes del envase, generalmente aceites esenciales, interaccionan durante el tiempo que el alimento está envasado, aprovechando estas propiedades antibacterianas y antioxidantes que presentan estos extractos naturales y aumentando así la durabilidad de los alimentos. Este nuevo concepto de envase en el que se fuerza la interacción del mismo con el alimento plantea una serie de retos, en relación a la evaluación de la seguridad, en comparación con el envasado tradicional que buscaba materiales inertes que actuasen como mera barrera frente al ambiente externo (Danielli y col., 2008).

En la actualidad, este tipo de envasado está siendo utilizado en determinados países como Japón y EE.UU.; sin embargo, la legislación europea, más restrictiva, ha retrasado su incorporación al mercado comunitario por demandar estudios que aseguren la utilidad e inocuidad de estos nuevos materiales. La nueva normativa comunitaria (EFSA, 2009b, 2011b; Reglamento UE 119/2011) intenta agilizar y sistematizar los procedimientos de presentación de solicitudes de autorización de sustancias activas, la evaluación de los riesgos inherentes a estos materiales y las normas relativas al etiquetado. Sin embargo, estos nuevos materiales son mínimamente utilizados por la industria alimentaria, pudiendo ser debido a factores socio-económicos, inquietudes y elecciones de los consumidores y, cuestiones no resueltas e inseguridades sobre la implementación (Chaves Sánchez, 2011).

Entre los constituyentes principales de estos nuevos envasados se encuentran los ya mencionados aceites esenciales, cuyo uso, con el fin de beneficiarse de sus propiedades antibacterianas, antioxidantes y/o aromáticas una vez que se ponen en contacto con los alimentos, se ha demostrado a lo largo de los años en muchas partes del mundo (Dainielli y col., 2008). Estos conocimientos previos sobre los aceites esenciales han hecho posible que aumente el interés del uso de los mismos, respondiendo a las demandas de la población y aumentando la vida útil de los alimentos, cubriendo debilidades presentes en la industria alimentaria. Los aceites esenciales y extractos de diferentes géneros de especies vegetales se han convertido, así, en un recurso a estudiar en el desarrollo de envases activos.

Muchos de estos aceites y sus componentes principales están recogidos en la Decisión de la Comisión 2002/113/EC como aromatizantes permitidos en la UE, por ejemplo el timol, linalol, carvacrol, limoneno o eugenol, para los que no se considera que exista riesgo para la salud del consumidor (Hyldgaard y col., 2012). En EE.UU., existen aceites esenciales (orégano, timo, mostaza o albahaca) que ya están clasificados como Generally Recognized as Safe (GRAS) por la FDA (Zhou y col., 2006; López y col., 2007). A parte de sus aplicaciones conocidas como aromatizantes se han desarrollado aplicaciones como conservantes en alimentación por las propiedades antioxidantes y antibacterianas que presentan (Dusan y col., 2006). Existen algunos trabajos en bibliografía que combinan la presencia de arcillas y aceites esenciales en matrices poliméricas, destacando la mejora de las propiedades barrera (causado por las arcillas) y el aumento de las propiedades antimicrobianas (debido a los aceites

esenciales de mejorana o clavo), dando lugar a un producto final con un perfil muy mejorado (Alboofetileh y col., 2014).

Teniendo en cuenta que la seguridad alimentaria y la calidad son las dos principales preocupaciones para la industria alimentaria, si el uso de estos aceites conduce a una mejora por su potencial antimicrobiano en el deterioro producido por microorganismos de los productos alimenticios y, consecuentemente, una disminución de diversas enfermedades en los seres humanos, estamos ante una opción muy atractiva. Además, la oxidación como proceso de degradación también juega un papel en numerosos productos alimenticios, siendo el potencial antioxidante de estas sustancias bien reconocido, presentándose así un papel crucial en este aspecto. Ortega-Ramírez y col. (2014) declararon que las propiedades antimicrobianas y antioxidantes de los compuestos bioactivos se deben principalmente a sus propiedades redox, su capacidad quelante de metales, y la capacidad de extinguir a especies reactivas de oxígeno singlete. Por esta razón, los aceites esenciales o sus componentes individuales son una buena opción para ser utilizados en la industria alimentaria para la mejora de la vida útil de los productos alimenticios. Sin embargo, hay algunas limitaciones para la aplicación de estas sustancias, ya que la variabilidad química en su composición podría desempeñar un papel importante en su actividad (Prakash y col., 2015).

Solórzano-Santos y Miranda-Novales (2012) publicaron que los efectos deseables (antimicrobiano/antioxidante) de los aceites esenciales o sus componentes añadidos directamente a los alimentos, se observaban a determinadas concentraciones, ya que a concentraciones superiores se producían cambios indeseables en el sabor, el olor, etc. Alcanzar concentraciones efectivas sin añadir grandes cantidades de estas sustancias ha llevado a la industria alimentaria a desarrollar diversos métodos de envasado activo en los que el aceite no es añadido como un ingrediente directo del alimento, sino como un componente del envase, produciéndose una liberación controlada de los mismos y ejerciéndose los efectos beneficiosos de manera gradual.

Con esta nueva propuesta de envasado alimentario, en los que se produce migración de las sustancias al espacio de cabeza y se genera inevitablemente contacto con el alimento, es necesario realizar una evaluación del riego exhaustiva para salvaguardar la salud del consumidor y cumplir así con el requisito de evaluación de la seguridad exigido por el Reglamento 1935/2004 y EFSA (2011b, 2015) sobre los materiales y

objetos destinados a entrar en contacto con alimentos. De tal forma, sería necesaria una evaluación caso por caso y una exhaustiva revisión bibliográfica de los estudios disponibles en bibliografía acerca del estado del arte de estos nuevos e interesantes sistemas de envasado activos, así como el riesgo asociado a la posible comercialización de estos envases.

III. JUSTIFICACIÓN Y OBJETIVOS/ SIGNIFICANT AND PURPOSES

Teniendo en cuenta lo descrito previamente, se deduce que aunque la aplicación de diferentes sustancias de origen natural incorporadas al envasado alimentario, tales como arcillas o aceites esenciales, es de interés con el fin de aumentar la vida útil del alimento, también supone un riesgo potencial para el hombre y el medio ambiente. Este riesgo está íntimamente ligado a la posible migración de dichos materiales incorporados a los polímeros de envase, que serían ingeridos junto con el alimento por el consumidor. De tal forma, una correcta evaluación de los riesgos derivados de la exposición humana a estas sustancias, arcillas o aceites esenciales, así como a los nuevos materiales de envasado que surgen tras su incorporación, se hace obligatoria. Esta evaluación requiere de una detallada caracterización del peligro, conociendo los efectos tóxicos que se producen tras la exposición de líneas celulares de órganos diana a los mismos, así como el resultado de la exposición de modelos experimentales *in vivo,* con el objetivo de poder extrapolar los datos de forma más certera al escenario de exposición humana real. En este sentido, tras una exhaustiva revisión bibliográfica en la que se verificó la disparidad de los resultados disponibles, se planteó el estudio de los efectos tóxicos de distintas organoarcillas modificadas con sales de amonio cuaternario y silanos, tanto *in vitro* como *in vivo*. De algunas de estas arcillas además, no existían datos en la literatura científica al ser de reciente desarrollo, por lo que su evaluación toxicológica se hacía más necesaria si cabe. Por otro lado, se evaluó *in vitro* el potencial genotóxico de compuestos mayoritarios del aceite esencial de orégano, una de las opciones más utilizadas en el área del envasado activo alimentario.

Teniendo en cuenta que la vía de exposición humana más probable es la oral, resulta de gran interés evaluar la respuesta de líneas celulares de órganos involucrados en el proceso digestivo, destacando el órgano primordial implicado en la absorción, el intestino, y el principal órgano implicado en la biotransformación de xenobióticos, el hígado. Por otro lado, sería interesante comprobar también el efecto tóxico tras la posible distribución a través de la circulación sanguínea de los materiales de estudio. Es por ello que se decidió investigar *in vitro*, en las células humanas Caco-2 (intestinales), HepG2 (hepáticas), y HUVEC (endoteliales), el potencial citotóxico y mecanismos de acción tóxica, a diferentes tiempos de exposición, de arcillas destinadas a ser incorporadas al envasado alimentario, así como el efecto a nivel molecular evaluando su expresión génica tras la exposición a concentraciones subcitotóxicas. Además, la evaluación toxicológica de extractos de migración obtenidos a partir del contacto de

simulantes alimentarios con los materiales nanocompuestos con arcillas incorporadas era necesaria, siendo evaluados éstos en diferentes modelos experimentales *in vitro*.

Una vez comprobado el potencial tóxico *in vitro* y seleccionada la arcilla de mejor perfil tecnológico y toxicológico, se consideró interesante llevar a cabo un estudio *in vivo* de toxicidad subcrónica a dosis respetidas (90 días) en roedores, basándonos en el peor escenario posible de migración al cual podría estar expuesto el consumidor. Se realizaron estudios histopatológicos en una amplia diversidad de órganos que podrían verse afectados y se evaluaron mediante diferentes ensayos marcadores de estrés oxidativo, de inflamación, expresión génica y abundancia proteica. Igualmente, se investigó la presencia de metales característicos de la estructura de las arcillas, ahondando más en la investigación para identificar posibles alteraciones.

En cuanto a los aceites esenciales y sus componentes mayoritarios los datos disponibles hasta el momento sobre su uso en el envasado de alimentos son contradictorios , , siendo necesaria también una amplia revisión de los principales efectos antimicrobianos/antioxidantes sobre el alimento proporcionados tras su incorporación al envase, y los principales efectos citotóxicos en líneas celulares humanas. Además, dentro de las apuestas de la industria alimentaria, destaca el aceite de orégano y sus componentes más importantes, el carvacrol y el timol. El uso de estos compuestos ha sido descrito por diversos autores pero los resultados publicados por el momento no indican un comportamiento similar de los mismos. Por ello, nos resultó interesante evaluar la genotoxicidad del carvacrol y timol mediante dos ensayos exigidos por la EFSA, el ensayo de micronúcleos y el ensayo de linfoma de ratón, el segundo de ellos no llevado a cabo hasta el momento, completando así la información toxicológica disponible de estas sustancias.

Para la realización de esta Tesis Doctoral, la doctoranda llevó a cabo dos estancias nacionales y una estancia internacional de investigación, en la que se abordaron tres objetivos fundamentales: a) Desarrollo y diseño de nuevas arcillas modificadas con silanos destinadas al envasado alimentario, así como un acercamiento a las técnicas de evaluación de propiedades tecnológicas: mecánicas, térmicas y de barrera; b) aprendizaje de nuevos ensayos de genotoxicidad de acuerdo con la normativa de la Organización para la Cooperación y Desarrollo Económico (OCDE), tales como el ensayo de micronúcleos y el ensayo de linfoma de ratón, exigidos por las autoridades

competentes para la comercialización de nuevos productos que van a estar en contacto con alimentos; c) aprendizaje de técnicas de biología molecular como aislamiento de ARN y PCR cuantitativa a tiempo real (q-PCR), investigando la influencia que tienen las arcillas destinadas al envasado de alimentos en la modulación de la expresión génica a nivel de ARNm.

Por todo ello, los objetivos específicos establecidos en la presente Tesis Doctoral han sido:

1. Realizar una exhaustiva valoración y revisión bibliográfica del estado del arte actual y los datos de toxicidad disponibles de arcillas no modificadas y modificadas destinadas a la industria alimentaria, así como de los materiales poliméricos resultantes que las contienen.
2. Evaluar el potencial tóxico *in vitro* de la arcilla no modificada, la montmorillonita (Cloisite®Na^+ (CNa^+)), y de arcillas modificadas con sales de amonio cuaternario (Cloisite®30B (C30B), Cloisite20A (C20A), Clay1 y Clay2) en líneas celulares de órganos diana (HepG2, Caco-2 y HUVEC), determinando los mecanismos de acción tóxica mediante la realización de ensayos de genotoxicidad/mutagenicidad, estrés oxidativo, inflamación, etc., y definiendo el perfil toxicológico de cada una, además de una evaluación mediante microscopía de posibles alteraciones celulares ultraestructurales.
3. Poner a punto e investigar por primera vez, la influencia que tienen las arcillas (CNa^+, C30B, Clay1 y Clay2) en la modulación de la expresión génica a nivel de ARNm de células HepG2 mediante la técnica q-PCR.
4. Estudiar el potencial citotóxico y mutagénico de los extractos de migración obtenidos a partir de polímeros (ácido poliláctico) con organoarcillas incorporadas (Clay1 y Clay2), además del contenido de metales (Al, Ca, Fe, Mg, Si) que hayan migrado del envase al simulante alimentario.
5. Evaluar el potencial tóxico *in vivo,* mediante un ensayo de toxicidad oral subcrónica de 90 días en roedores (rata Wistar), de una arcilla modificada (Clay1) y su extracto de migración, investigando la inducción de estrés oxidativo como mecanismo de acción tóxica, los cambios y alteraciones histopatológicas de distintos órganos tras la exposición, marcadores de inflamación y análisis de la bioquímica clínica.

6. Explorar las alteraciones a nivel de expresión génica mediante qPCR y Western Blot de enzimas involucradas en la defensa antioxidante (CAT y SOD) en órganos diana (hígado y riñón) tras la exposición subcrónica de roedores a la arcilla modificada Clay1 y su extracto de migración.
7. Analizar la presencia de metales comunes de la estructura de las arcillas en el bazo de roedores, tras la exposición durante 90 días a la arcilla modificada Clay1 y su extracto de migración.
8. Desarrollo y caracterización de tres nuevas arcillas modificadas con silanos (Clay3, Clay4A y Clay4B) destinadas a ser incorporadas a polipropileno con el fin de mejorar sus propiedades para su aplicación en envasado alimentario y evaluación del incremento de la vida útil de alimentos.
9. Investigar el potencial tóxico *in vitro* de las nuevas arcillas modificadas con silanos y sus extractos de migración en dos líneas celulares de órganos diana (HepG2 y Caco-2) mediante ensayos de citotoxicidad, genotoxicidad/mutagenicidad, estrés oxidativo y citometría de flujo.
10. Evaluar la migración de metales propios de la estructura de las arcillas en simulantes alimentarios que han estado en contacto con materiales nanocompuestos poliméricos conteniendo Clay3 y Clay4B.
11. Revisar la bibliografía disponible sobre los efectos antioxidantes y antimicrobianos de los aceites esenciales y sus componentes mayoritarios incorporados a envases y las propiedades de los envases resultantes, así como llevar a cabo un estudio de sus principales efectos citotóxicos.
12. Estudiar la genotoxicidad de los compuestos mayoritarios del aceite esencial de orégano, timol y carvacrol, mediante el ensayo de micronúcleos y, por primera vez, el ensayo de linfoma de ratón.

El trabajo experimental se ha realizado en el Área de Toxicología de la Facultad de Farmacia de la Universidad de Sevilla, haciendo uso, así mismo, del Servicio de Biología y de Microscopía del Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS). El desarrollo de las arcillas se ha llevado a cabo en colaboración con el Área de Materiales del Instituto Tecnológico del Embalaje, Transporte y Logística (ITENE) de Valencia, gracias a las Dras. Susana Aucejo y María Jordá. Parte de la investigación llevada a cabo a nivel molecular, así como el ensayo de

genotoxicidad de micronúcleos en células HepG2, fueron llevados a cabo en el Departamento de Toxicología Genética y Biología del Cáncer, del Instituto Nacional de Biología (NIB) de Ljubljana (Eslovenia), bajo la dirección y supervisión de la Dras. Metka Filipic y Bojana Zegura. El análisis morfológico a nivel celular ha sido realizado en colaboración con el Dr. Javier Moreno Onorato del grupo de Citoquímica Ultraestructural del Departamento de Biología Celular de la Facultad de Biología de la Universidad de Sevilla. Así mismo, los estudios histopatológicos se han realizado en colaboración con el Área de Toxicología y el Departamento de Anatomía y Anatomía Patológica Comparadas, ambos de la Universidad de Córdoba, gracias a la colaboración de la Dra. Rosario Moyano y el Dr. Alfonso Blanco. La evaluación de la bioquímica clínica del suero sanguíneo se ha llevado a cabo en la Unidad de Bioquímica Clínica del Hospital Universitario Virgen Macarena de Sevilla. Para el aprendizaje de los ensayos de MN y linfoma de ratón se contó con la colaboración del Grupo de Mutagénesis del Departamento de Genética y Microbiología de la Universidad Autónoma de Barcelona bajo la dirección del Dr. Ricard Marcos.

Siguiendo la normativa de la Universidad de Sevilla, el resumen, la justificación y objetivos, y las conclusiones se redactan tanto en español como en inglés para optar a la "Mención Internacional en el Título de Doctor".

Taking into account the state of the art previously described, it is important to note that although the new incorporation of natural substances, such as clays or essential oils, into food packaging has a great interest in order increase the shelf life of food, they also poses a potential risk for humans and the environment. This risk is closely linked to the possible migration of the materials incorporated into packaging polymers, which would be ingested with food by the consumer. Therefore, a comprehensive assessment of human exposure risks to these substances, clays or essential oils, together with the new packaging materials created with them, is mandatory. This assessment requires a detailed characterization of the hazard, exploring the toxic effects that occur after exposure of several cell lines from target organs, as well as, the result of the exposure with *in vivo* experimental models, in order to extrapolate the data obtained to real human exposure scenario. In this sense, after a thorough literature review in which the disparity of the available results has been verified, the study of the toxic effects of different organomodified clays with quaternary ammonium salts and silanes, both *in vitro* and *in vivo*, has been performed. In addition, some of these clays have been recently developed, therefore no available data in the literature have been found, being the toxicological evaluation even more necessary. On the other hand, *in vitro* genotoxic potential of major compounds of the oregano essential oil was evaluated, being one of the most used essential oils in active food packaging.

In this sense, considering that the oral route is the most important to human exposure in this kind of substances, it is of great interest to evaluate the response of target cell lines from organs involved in the digestive process, emphasizing the first organ involved in the absorption, intestine, and the main organ involved in xenobiotics biotransformation, liver. On the other hand, it would be also interesting to check the toxic effect after a possible distribution through the bloodstream of the materials under study. We decided to investigate *in vitro*, in human cell lines such as Caco-2 (intestinal), HepG2 (liver), and HUVEC (endothelial), the cytotoxic potential and the main mechanisms of toxicity at different exposure times of several clays intended to be incorporated into food packaging. Moreover, the effects produced at the molecular level were also evaluated through the gene expression analysis at subcytotoxic concentrations. In addition, the toxicological evaluation of migration extracts obtained from polymer-clay nanocomposites was necessary, being evaluated in different *in vitro* experimental models.

After the *in vitro* toxicity approach, the clay with the best technological and toxicological profile was selected to perform a repeated dose 90-day oral toxicity study in Wistar rats, based on the worst scenario of migration which could be exposed the consumer. Histopathological studies were performed in a wide variety of organs that could be affected and different markers of oxidative stress, inflammation, gene expression and protein abundance were evaluated. Similarly, the presence of the major metal present in clays structure was determined in order to identify other possible alterations.

Regarding to the essential oils and their main components the available data on their use in food packaging, are contradictory so far, being necessary an exhaustive review of the main antimicrobial/antioxidant effects of the resultant active packaging on the food, and the main cytotoxic effects on human cell lines. Moreover, oregano essential oil and its major components, carvacrol and thymol, are one of the options most used in the food industry. The use of these compounds has been described by several authors but the results published do not indicate a similar behavior of them so far. Therefore, we found it interesting to evaluate the genotoxicity of carvacrol and thymol by two tests required by EFSA, the micronucleus test and mouse lymphoma assay, the second one has not been carried out until now, being completed the available toxicological information of these substances.

For the fulfillment of this thesis, the PhD student performed internships, two nationals and one international, in which several objectives were addressed: a) development and design of new silane-modified clays intended to be used in food packaging as well as an approach to techniques to evaluation their technological properties: mechanical, thermal and barrier; b) learn new genotoxicity tests according to the rules of the Organization for Economic Cooperation and Development (OECD), such as the micronucleus test and mouse lymphoma assay, required by the competent authorities to commercialize new products that will be in contact with food; c) learn molecular biology techniques as RNA isolation and real-time quantitative PCR (q-PCR), to investigate the influence of clays in the modulation of gene expression at the mRNA level.

Therefore, the specific objectives in this PhD thesis were:

1. To perform a comprehensive assessment and literature review of the current state of art and toxicity data of unmodified and modified clays intended to be used in the food industry, as well as, the resulting polymeric materials that contain them.
2. To evaluate the *in vitro* toxicity potential of the unmodified clay, montmorillonite (Cloisite®Na^+(CNa^+)) and quaternary ammonium salts-modified clays (Cloisite®30B (C30B), Cloisite20A (C20A) and Clay2 clay1) in target cell lines (HepG2, Caco-2 and HUVEC), determining the action mechanisms by testing genotoxicity/mutagenicity, oxidative stress, inflammation, etc., defining each toxicological profile, and possible ultrastructural cellular alterations by microscopy.
3. To develop and investigate the influence of clays (CNa^+, C30B, Clay1 and Clay2) in the modulation of gene expression at the mRNA level of HepG2 cells by q-PCR.
4. To study the cytotoxic and mutagenic potential of migration extracts obtained from polymers (polylactic acid) with organomodified clays (Clay1 and Clay2) and the migration of metals content (Al, Ca, Fe, Mg, Si) presented in the food simulants.
5. To analyze the *in vivo* toxicity potential, by performing a repeated dose 90-day oral toxicity study in Wistar rats exposed to a modified clay (Clay1) and its migration extract, researching the induction of oxidative stress as a toxicity mechanism, histopathological alterations of different organs, inflammation responses and analysis of clinical biochemistry.
6. To explore the alterations in gene expression by qPCR and Western blotting of enzymes involved in antioxidant defense (SOD and CAT) from target organs (liver and kidney) of rats exposed subchronically to modified clay, Clay1, and its migration extract.
7. To determine the presence of major metals of clays structure in the spleen of rodents after exposure during 90 days to a modified clay, Clay1, and its migration extract.
8. To develop and characterize three new silane-modified clays (Clay3, Clay4A and Clay4B) intended to be incorporated to polypropylene (PP) in order to improve their properties for application in food packaging and evaluation of the increase of food shelf life.

9. To investigate the *in vitro* toxicity potential of new silane-modified clays and their migration extracts in two target cell lines (HepG2 and Caco-2) by testing cytotoxicity, genotoxicity / mutagenicity, oxidative stress and flow cytometry.
10. To evaluate the metals migration presented in clays structure in food simulants which have been in contact with Clay3 and Clay4A polymer nanocomposites.
11. To review the available literature on antioxidants and antimicrobial effects of essential oils and their major components incorporated into packaging and properties of the resulting packages, as well as, conducting a study of their main cytotoxic effects.
12. To study the genotoxicity of the main compounds of oregano essential oil, thymol and carvacrol, using the micronucleus test and, for the first time, the mouse lymphoma assay.

The experimental work has been performed in the Area of Toxicology, Faculty of Pharmacy, University of Sevilla, using also the Biology Services from Centro de Investigación, Tecnología e Innovación from the University of Sevilla (CITIUS). The development of the clays was carried out in collaboration with the Department of Materials from the Technological Institute of Packaging, Transport and Logistics (ITENE) from Valencia, thanks to Drs. Susana Aucejo and Maria Jorda. Part of the research conducted at the molecular level, as well as, genotoxicity testing of micronuclei in HepG2 cells were carried out in the Department of Genetic Toxicology and Cancer Biology, National Institute of Biology (NIB) to Ljubljana (Slovenia), under the direction and supervision of Drs. Metka Filipic and Bojana Zegura. Cellular morphological analysis has been conducted in collaboration with Dr. Javier Moreno Onorato, belonging to Ultrastructural Cytochemistry group from the Department of Cell Biology, Faculty of Biology, University of Sevilla. Histopathological studies have been performed in the Area of Toxicology and in the Department of Anatomy and Comparative Pathology and Anatomy, both from the University of Córdoba, thanks to the collaboration of Dr. Rosario Moyano and Dr. Alfonso Blanco, respectively. The clinical biochemistry evaluation of blood serum has been carried out in the Clinical Biochemistry Unit of the University Hospital Virgen Macarena in Seville. MN and mouse lymphoma assays were learned thanks to the collaboration with the Group of Mutagenesis of the Department of Genetics and Microbiology at the University Autónoma of Barcelona under the direction of Dr. Ricard Marcos.

Following the regulations from the University of Sevilla, the summary, significance and purposes, and conclusions have been written both in Spanish and English to aim for a PhD with International Mention.

IV. RESULTADOS Y DISCUSIÓN/ RESULTS AND DISCUSSION

CAPÍTULO 1 / CHAPTER 1

Sara Maisanaba, Silvia Pichardo, María Puerto, Daniel Gutiérrez-Praena, Ana M. Cameán, Ángeles Jos

TOXICOLOGICAL EVALUATION OF CLAY MINERALS AND DERIVED NANOCOMPOSITES: A REVIEW

Environmental Research 138, 233-254, ***2015***

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Review

Toxicological evaluation of clay minerals and derived nanocomposites: A review

Sara Maisanaba, Silvia Pichardo, María Puerto, Daniel Gutiérrez-Praena, Ana M. Cameán, Angeles Jos*

Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González 2, 41012 Sevilla, Spain

ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form
22 December 2014
Accepted 24 December 2014

Keywords:
Clay mineral
Toxicity
In vitro
In vivo
Nanocomposites

ABSTRACT

Clays and clay minerals are widely used in many facets of our society. This review addresses the main clays of each phyllosilicate groups, namely, kaolinite, montmorillonite (Mt) and sepiolite, placing special emphasis on Mt and kaolinite, which are the clays that are more frequently used in food packaging, one of the applications that are currently exhibiting higher development. The improvements in the composite materials obtained from clays and polymeric matrices are remarkable and well known, but the potential toxicological effects of unmodified or modified clay minerals and derived nanocomposites are currently being investigated with increased interest. In this sense, this work focused on a review of the published reports related to the analysis of the toxicological profile of commercial and novel modified clays and derived nanocomposites. An exhaustive review of the main *in vitro* and *in vivo* toxicological studies, antimicrobial activity assessments, and the human and environmental impacts of clays and derived nanocomposites was performed. From the analysis of the scientific literature different conclusions can be derived. Thus, *in vitro* studies suggest that clays in general induce cytotoxicity (with dependence on the clay, concentration, experimental system, *etc.*) with different underlying mechanisms such as necrosis/apoptosis, oxidative stress or genotoxicity. However, most of *in vivo* experiments performed in rodents showed no clear evidences of systemic toxicity even at doses of 5000 mg/kg. Regarding to humans, pulmonary exposure is the most frequent, and although clays are usually mixed with other minerals, they have been reported to induce pneumoconiosis *per se*. Oral exposure is also common both intentionally and unintentionally. Although they do not show a high toxicity through this pathway, toxic effects could be induced due to the increased or reduced exposure to mineral elements. Finally, there are few studies about the effects of clay minerals on wildlife, with laboratory trials showing contradictory outcomes. Clay minerals have different applications in the environment, thus with a strict control of the concentrations used, they can provide beneficial uses.

Despite the extensive number of reports available, there is also a need of systematic *in vitro–in vivo* extrapolation studies, with still scarce information on toxicity biomarkers such as inmunomodulatory effects or alteration of the genetic expression. In conclusion, a case by case toxicological evaluation is required taking into account that different clays have their own toxicological profiles, their modification can change this profile, and the potential increase of the human/environmental exposure to clay minerals due to their novel applications.

1. Introduction

Clays and clay minerals (Table 1) belong to the phyllosilicate group (from the Greek “phyllon”: leaf, and from the Latin “silic”: flint) (Fig. 1). As a distinctive feature, these materials are very small (a few micrometres maximum), and their preferred formation occurs under the surface (alterites, soils, and sediments) or subsurface (diagenesis and hydrothermal alterations) conditions (Meunier, 2005). The physical and chemical properties of a particular clay mineral are dependent on its structure and composition. The structure and composition of the main industrial clays, *i.e.*, kaolins (1:1 phyllosilicates), smectites (2:1 phyllosilicates) and sepiolite (2:1 inverted ribbons), are very different (Table 2), even though each is composed of octahedral and tetrahedral sheets as their basic building blocks. However, the arrangement and composition of the octahedral and tetrahedral sheets account for most of the differences in their physical and chemical properties (Murray, 2007a).

* Corresponding author. Fax: +34 954 556422.
E-mail address: angelesjos@us.es (A. Jos).

http://dx.doi.org/10.1016/j.envres.2014.12.024
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Table 1
Diferences between clay and clay mineral (Bergaya and Lagaly, 2013).

Clay	Clay mineral
Natural	Natural and synthetic
Fine-grained (<2 or <4 μm)	No size criterion
Phyllosilicates as principal constituents	May include non-phyllosilicates
Plastic (with some exceptions like flint clays)	Plastic
Hardens on drying or firing	Hardens on drying or firing

Clays and clay minerals, either as such or after modification, are recognized as the materials of the 21st century because they are abundant, inexpensive and environmentally friendly. Further introductory aspects of clays have been described by Bergaya and Lagaly (2013). Phyllosilicates are very important industrial minerals. There are well over 100 documented industrial applications of clay materials. Clays are utilized in the process industries, in agricultural applications, in engineering and construction applications, in environmental remediation, in geology, and in many other miscellaneous applications (Murray, 2007b). Currently, the use of several clays in the food industry is a reality for improving food packaging. This review focused on the main clays of each phyllosilicate group, namely kaolinite, montmorillonite (Mt) and sepiolite, and placed special emphasis on Mt and kaolinite, which are the clays that are more frequently used in food packaging.

These two clay minerals are among the most frequently used solid fillers for obtaining novel polymer composites, named nanocomposites, due to the small quantity of clay minerals that is added to the polymer matrix to control the final properties of the nanocomposite and to yield better workability (Annabi-Bergaya 2008; Arora and Padua, 2010). The octahedral and tetrahedral sheets of their structure, which are known as platelets, are approximately 1 nm thick and 100 to 500 nm in diameter, resulting in platelets with a high aspect ratio (Uyama et al., 2003), but clays are formed by numerous platelets, yielding submicrometer particles. When clays are dispersed into a polymer matrix, they experience swelling, dividing all of the platelets into independent units at the nanometre scale, hence the name "nanocomposite materials". These platelets force gases to follow a tortuous path through the material which greatly slows their transmission. As a result, a nanolayer clay structure is obtained, and the path of diffusion of the penetrating molecules of gases and other substances increases, providing substantially improved products (Abacha et al., 2009; Choudalakis and Gotsis, 2009; Herrera-Alonso et al., 2010). The effect of nanoclays on the polymer properties lies mainly on their high surface-to-volume ratio because polymer–filler interactions are governed by interfacial forces (Arora and Padua, 2010). The successful formation of a polymer–clay nanocomposite relies on two key characteristics of the component used for reinforcement. The first characteristic is the ability to modify the chemical surface of the silicates through ion-exchange reactions with organic and inorganic cations in order to produce a polymer-compatible nanocomponent. The second characteristic is the ability of the silicate particles to disperse into the polymer, yielding an exfoliated nanocomposite. In this regard, three different clay dispositions can be obtained: (1) Tactoid structures remain in a polymer when the interlayer space of the clay gallery does not expand, usually due to its poor affinity with the polymer. No true nanocomposites are formed this way (Alexandre and Dubois, 2000). (2) Intercalated structures are obtained at moderate expansion of the clay interlayer. In this case, interlayer spaces expand slightly as polymer chains penetrate the basal spacing of clay, but the shape of the layered stack remains. This is the result of moderate affinity between polymer and clay. (3) Exfoliated structures, clay clusters lose their layered identity and are well separated into single sheets within the continuous polymer phase. This is due to a high affinity between polymer and clay (Arora and Padua, 2010) (Fig. 2). These two characteristics are, of course, connected to each other because the degree of dispersion of a layered silicate in a particular polymer matrix depends on the interlayer cation modification (Ray and Okamoto, 2003). However, a simple dispersion of clays in a polymer matrix will not produce a nanocomposite with better properties compared with those of the bulk material due to the poor interfacial interactions between the hydrophilic reaction sites of clays and the highly hydrophobic polymer chains (Pisticelli et al., 2010). The solution for the aforementioned disadvantages can be achieved by introducing organic groups to enhance the interaction between the clays and polymers (Silva et al., 2011). In this sense, cation exchange is one of the most useful chemical modification procedures used to prepare more organophilic clays (Pavlidou and Papaspyrides, 2008; Paul and Robenson, 2008; Ray and Okamoto, 2003), and one of the modifiers that is most frequently used is quaternary ammonium salts (Fig. 3). This produces modified clays with a hydrophobic character and a greater interlayer space (Betega de Paiva et al., 2008; Jordá-Beneyto et al., 2014). Several quaternary ammonium salt-modified clays are available in the market, and other clays are under development; thus, all of these result in recognized improvements to the polymers (Bin-Haron, 2010; Carreu, 2011; Jordá-Beneyto et al., 2014; Lai and Kim, 2005; Pal et al., 2014).

Although the beneficial technical effects of the nanocomposites obtained from clay minerals have been known for years and are generally well described, the potential toxicological effects and impacts of unmodified or modified clay minerals and derived nanocomposites on human and environmental health are

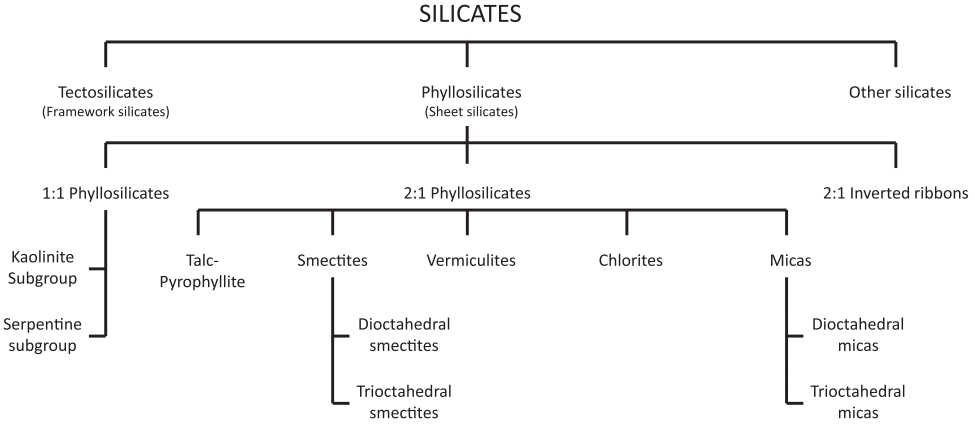

Fig. 1. Classification of silicates with the main subgroups of clays (WHO, 2005).

Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

Table 2
Chemical composition of the main clays cited.

Name	Chemical composition	
	Basic chemical formula	Modifier and modifier concentration
Cloisite® Na^+	Montmorillonite(Mt) $Na_{0.2}Ca_{0.1}Al_2Si4O_{10}(OH)_2(H2O)_{10}$	None
Cloisite® 30B	Modified Mt	Quaternary ammonium salt (MT2EtOH) 90 meq/100 g clay
Cloisite® 20A	Modified Mt	Quaternary ammonium salt (2M2HT) 95 meq/100 g clay
Cloisite® 93A	Modified Mt	Ternary ammonium salt (M2HT) 90 meq/100 g clay
Cloisite® 15A	Modified Mt	Quaternary ammonium salt (2M2HT) 125 meq/100 g clay
Cloisite® 10A	Modified Mt	Quaternary ammonium salt (2MBHT) 125 meq/100 g clay
Clay1	Modified Mt	Quaternary ammonium salt (HDTA) 6-fold the CEC of raw clay (raw clay with a cation exchange capacity (CEC)=92.6 meq/100 g clay)
Clay2	Modified Mt	Quaternary ammonium salt (HDTA+ACO) HDTA in 5.75-fold and ACO in 0.25-fold of the CEC of raw clay (92.6 meq/100 g clay)
Oligo(styrene-co-acrylonitrile) Mt	Modified Mt	Quaternary ammonium salt of poly(styrene-co-acrylonitrile) (CEC=0.9meg/g clay)
Bentonite	Non-modified $Al_2O_34SiO_2H_2O$	None
Active bentonite	Modified bentonite	H_2SO_4 10–15%
Kaolinite	$Al_2Si_2O_5(OH)_4$	None
Sepiolite	$Mg_4Si_6O_{15}(OH)_2 \cdot 6H_2O$	None
Erionite	$(Na_2,K_2,Ca)_2Al_4Si_{14}O_{36} \cdot 15H_2O$	None
Halloysite	$Al_2Si_2O_5(OH)_4$	None
Hectorite	$Na_{0.3}(Mg,Li)_3Si_4O_{10}(OH)_2$	None
Illite	$(K,H_3O)(Al,Mg,Fe)_2(Si,Al)_4O_{10}[(OH)_2,(H2O)]$	None

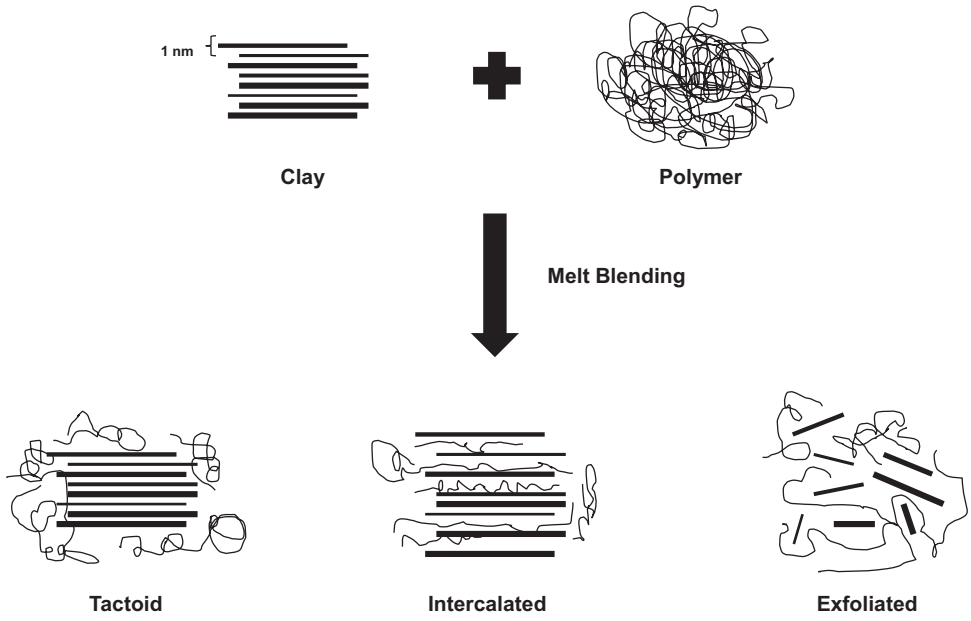

Fig. 2. Schematic representation of intercalated and exfoliated nanocomposites from layered silicate clay filler and polymer (based on Arora and Padua, 2010).

currently being investigated with increased interest. The general population exposure to low concentrations of Mt, kaolinite, and other clay minerals in their natural form is ubiquitous (WHO, 2005). The incorporation of the clay minerals into polymers can result in non-intentional exposure to the consumer, and it is thus necessary to evaluate not only the microstructured clay but also the imbued nanomaterial in the polymer due to the possible migration to the food product (EFSA, 2011a; Guillard et al., 2010; Song et al., 2011). Moreover, humans can absorb clays through occupational and environmental exposures and also due to their presence in consumer products (pharmaceutical formulations, spas, and beauty therapy) (Carretero et al., 2006). In contrast, the presence of clays in the environment and their effects on wildlife can also be derived from intentional and non-intentional sources.

The toxicological evaluation of these products can be assessed using both *in vitro* and *in vivo* methods. Several researchers have already evaluated the *in vitro* toxicological profiles of different clays and derived nanocomposites, concluding that the toxicity shown depends on various parameters, the most important of which were the modifiers used, the experimental model selected, the concentration range and the exposure times (Section 2). Regarding the *in vivo* assessments, although it has been recognized that the interaction of clays with organs may provoke health-deleterious consequences, to date, there is little mechanistic understanding of the physiological effects due to exposure to clays (Kibanova et al., 2009). Some studies performed *in vivo* can be found in the literature, and different results have been published (Section 3). Additionally, an evaluation of the impact of these materials on human and environmental health is also necessary, and different studies are available on this topic (Section 4).

Thus, this work focused on the review of the published reports related to the analysis of the toxicological profiles of commercial and novel modified clays and derived nanocomposites. A review of the main *in vitro* and *in vivo* toxicological studies, antimicrobial

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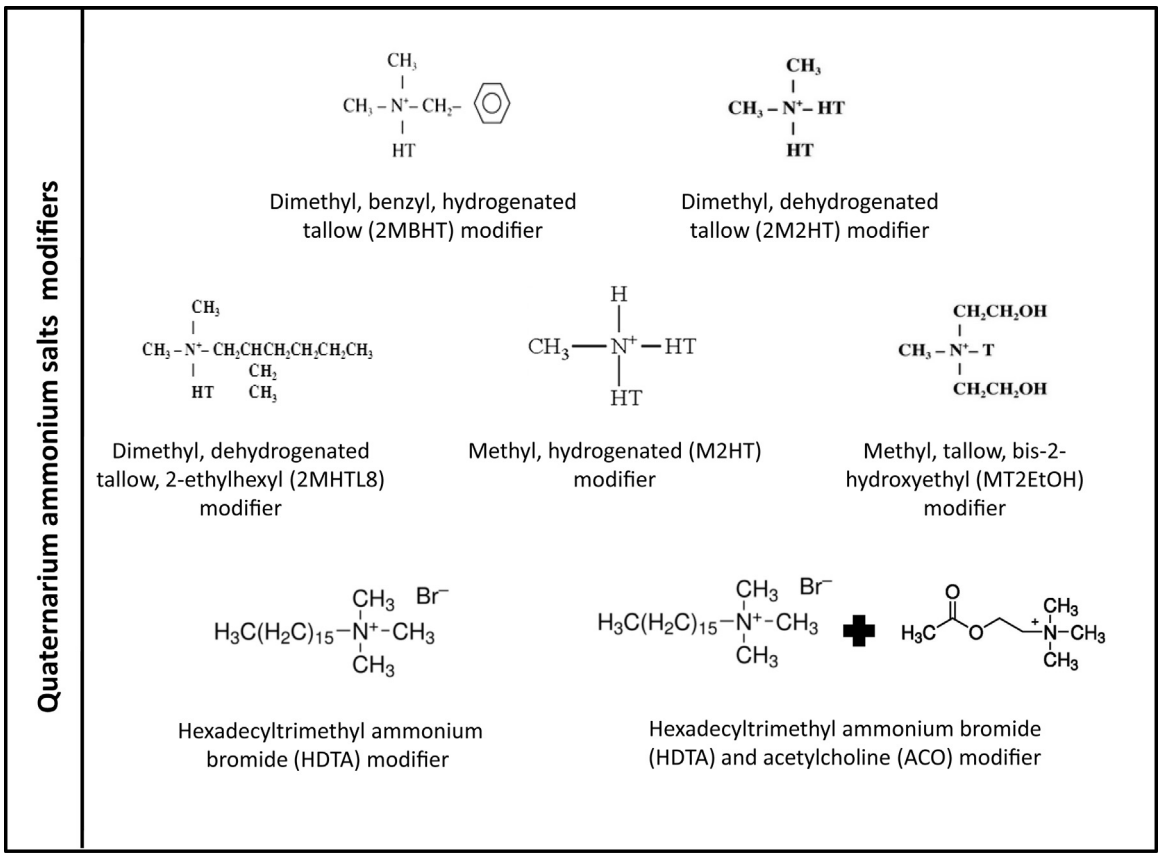

Fig. 3. Commercial quaternary ammonium salts modifiers used in modified clays.

activity assessments, and the human and environmental impacts of clays and derived nanocomposites was performed. The information used in the review was gathered through an extensive search of different sources in the public domain (scientific literature from PubMed and Sciencedirect, reports from EU/international authorities and various other sources). Priority was given to information from peer-reviewed sources; however, other sources of available data were also taken also into consideration when relevant. The main objectives were therefore to identify the state-of-the-art information on the toxicological profile of clays and derived composites and to identify possible data gaps and research needs in this field.

2. *In vitro* toxicological assays

In vitro systems are mainly used for screening purposes and for generating more comprehensive toxicological profiles (Eisenbrand et al., 2002).

Several toxicity endpoints can be determined through *in vitro* methods, providing evidence of the basal damage or the mechanism of action responsible of the toxic insult. The *in vitro* toxicity of clay minerals has been widely studied in the scientific literature, and the main findings are described below.

2.1. Basal cytotoxicity assays and morphological studies

Many cell lines have been used to study the toxicity induced by clays and clay-containing nanocomposites. A previous study performed by the World Health Organization (WHO) reviewed the effects on an *in vitro* system of bentonite, kaolin and other clay minerals (WHO, 2005). However, the works referred are dated to the period 1969–1996. In the last four years, studies in this field have substantially increased; in this respect, the most recent studies are summarized in Table 3. A wide variety of clay minerals have been studied, from platelet nanoclays, such as Mt, bentonite, kaolinite and erionite, to clay nanotubes, such as halloysite, both in pure form and included in polymeric matrices. Human cell lines are the most frequently used experimental model, although some from rodents have also been employed. In regard to the cell type, intestine and liver cells have been widely used, followed by lung and skin cell lines, evidencing the interest for the use of these compounds in the food industry as well as the relevance of their environmental impact.

Unmodified Mt, including bentonite, has been reported to cause cytotoxicity in many cell lines. In this sense, Murphy et al. (1993a) evidenced a decrease in cell viability in HUVECs exposed for 24 h to 0.1 mg/mL bentonites, although limited results were obtained in the neuronal cell lines N1E-115 and ROC-1. Similarly, CHO cells exposed to 1 mg/mL Mt for up to 24 h underwent a reduction in cell viability (Li et al., 2010). Baek et al. (2012) reported similar results in intestinal cells (INT-407) exposed to the same concentration of Mt, showing a concentration- and time-dependent inhibition of cell proliferation after 24–72 h of exposure, as well as cytotoxicity after long-term exposure to Mt. It has been suggested that the organo-modifier included in the Mt modulates the toxicity of the resulting clay (Sharma et al., 2010). In this sense, Cloisite®Na^+ (CNa^+) did not show signs of cytotoxicity in Caco-2 cells, although Cloisite®30B (C30B) induced approximately 40% cytotoxicity at the highest concentration tested (226 μg/mL) after 24 h of exposure (Sharma et al., 2010). In addition, the cytotoxicity induced by CNa^+ on HepG2 cells was lower than that obtained after exposure to Cloisite®93A (C93A) for 24 h (Lordan et al., 2011). In addition, active bentonite, which is bentonite that is industrially treated with H_2SO_4, produced greater damage in human B lymphoblast cells than the native bentonite

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Table 3
Cytotoxic effects of unmodified/modified clays and derived nanocomposites.

Material tested	Modifier	Experimental model	Assays performed	Concentration range	Exposure time	Main results	Reference
- Sepiolite	Unmodified	Primary rat hepatocytes	Lactate dehydrogenase (LDH) release assay	1-10 μg/mL	20 h	No significant differences were observed in comparison with the controls.	Denizeau et al. (1985)
- Mt, bentonite, kaolinite and erionite	Unmodified	HUVE, N1E-115 and ROC-1 cell lines	LDH release assay, Trypan Blue exclusion test	0.1 mg/mL	24 h	Only HUVE cells experienced significant LDH release after exposure to bentonite, kaolinite and Mt. Similarly, all clays changed significantly cell viability in HUVE cells, but only Mt and erionite did in N1E-115. No effect was reported in ROC-1 cells	Murphy et al. (1993a)
- Quartz – Kaolin (Both untreated and treated)	Dipalmitoyl phosphatidylcoline (DPPC)	Rat pulmonary alveolar macrophages cells	Live-Dead assay	0-80 μg/cm^2 0-40 μg/cm^2	1, 3, 5 days	Concentration and time cytotoxicity was observed in cells exposed for 1, 3 and 5 days to untreated quartz and kaolin. DPPC-surfactant pretreatment delayed the toxic effects	Gao et al. (2000)
-Native Bentonite - Modified Bentonites	Unmodified α-quartz+chemical modifications (alkalin, acid and organic)	IMR90 cell line	Alarm Blue assay	1-50 μg/cm^2	24 h	All bentonite samples induced cytotoxic effects, being more evident in bentonites with higher quartz contents than in untreated bentonites or those with lower quartz content	Geh et al. (2006)
- Native Bentonite (BPN) - Active Bentonite (BPA)	Unmodified H_2SO_4	HMy2.CIR cell line	Cell Counting, neutral red uptake, LDH release assay	0-1000 μg/mL	4 and 24 h	Cell viability decrease with the exposure concentrations and time. BPA being more cytotoxic than BPN	Meibian et al. (2010)
- Mt	Unmodified	CHO cell line	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, LDH release	0-1000 μg/mL	3, 12, 24 h	Reduction of 40% cell viability and increase in 40% in LDH levels after 24 h of exposure to 1000 μg/mL Mt	Li et al. (2010)
- CNa^+ - C30B (Unfiltered and filtered)	Unmodified Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	Alarmar Blue assay	0-226 μg/mL	24 h	Only the highest concentration used (226 μg/mL) of the unfiltered exposure underwent significant reduction in cell viability (40%)	Sharma et al. (2010)
- Halloysite Clay Nanotubes (HNT) unmodified and functionalized	Unmodified Aminopropyltriethoxysilane	HeLa and MCF-7 cell line	MTT assay, Trypan Blue assay	1-1000 μg/mL	24, 48, 72 h	Both HNTs exhibit growth inhibition in a concentration and time dependent manner at both cell lines and assays. The cell viability was preserved up to 75 μg/mL. No effect of functionalization was recorded	Vergaro et al. (2010)
- Mt (Na-MMT) - Oligo(styreme-co-acrylonitrile)-modified Mt (PSAN-MMT)	Unmodified Oligo(styrene-co-acrylonitrile)	HEK 293 and NIH 3T3 cell lines	MTT assay, LDH release	0.1–2 g/L	24 h	Significant cell viability decrease was observed in both cell lines exposed to both Mt in the LDH release assay and MTT assay. PSAN-MMT was less toxic than MMT	Liu et al. (2011)

Table 3 (*continued*)

Material tested	Modifier	Experimental model	Assays performed	Concentration range	Exposure time	Main results	Reference
- CNa^+ - C93A	Unmodified Ternary ammonium salt (M2HT)	HepG2 cell line	MTT assay, LDH release	0–1000 μg/mL	24 h	Concentration-dependent effect was observed in cells treated with both clays, being significant from 1 μg/mL in the MTT assay and from 50 μg/mL in the LDH release assay	Lordan et al. (2011)
- Aminopropyl magnesium phyllosilicate (AMP) - Aminopropyl calcium phyllosilicate (ACP)	Aminopropyl	CCD-986sk, A549, MRC-5, HT-29 cell lines	MTT assay, LDH release	1–1000 μg/mL	24, 48, 72 h	Decrease in cell viability and membrane damage appeared at the highest concentrations of both organoclays assayed	Han et al. (2011)
- Nanocomposite of halloysite clay nanotubes (HNT) incorporated into chitosan	Unmodified	NIH3T3 cell line	MTT assay	2, 5, 7.5 and 10%	1, 3 and 7 days	Both chitosan and chitosan/HNTs nanocomposites films are biocompatible since no visible reduction in viability was found	Liu et al. (2012)
- Mt	Unmodified	INT-407 cell line	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium (WST-1) assay, MTT assay, LDH release assay	0-1000 μg/mL	24, 48, 72 h and 10 days	Cell proliferation was inhibited in a concentration and time dependent in the short-term WST-1 assay. Similarly, after 10 days of exposure, all concentrations tested significantly reduced cell viability. Only 1000 μg/mL induced a significant release of LDH	Baek et al. (2012)
- Platelet nanoclays (Bentone MA, ME-100, Cloisite Na+, Nanomer PGV, Delite LVF) Tubular nanoclays (Halloysite and Halloysite MP1)	Not specified	A549 cell line	High content screening and real-time impedance sensing	1–250 μg/mL	24 h	No toxic effect was recorded for nanoclays up to 10 μg/mL, except for Delite LVF. At higher concentration, cells grow decreased in a concentration dependent manner. Tubular nanoclays showed lower cytotoxicity compared to platelet type, with Bentone MA and ME-100 showing the better results in this group	Verma et al. (2012)
- Nanocomposites of an unmodified sepiolite, unmodified and modified Mt and fluorohectorites	Poly(butylene adipate-co-terephthalate) and its nanocomposites based on 10 wt.% clay minerals. Modified Mt contained a quaternary ammonium salt (MT2EtOH), and modified fluorohectorite contained a dihydroxy organic modifier.	L929 cell line	MTT assay	10 wt% clay minerals	1, 2 and 5 days	No visible reduction in viability was observed at any experimental condition.	Fukushima et al. (2012)
- Nanocomposites of an unmodified sepiolite, unmodified and modified Mt and fluorohectorites	Poly(butylene adipate-co-terephthalate) and its nanocomposites based on 10 wt.% clay minerals. Modified Mt contained a quaternary ammonium salt (MT2EtOH), and modified fluorohectorite contained a dihydroxy organic modifier.	Fibroblast and osteoblast cell lines	MTT assay	10 wt% clay minerals	1, 4 and 7 days	No significant reduction in viability was observed in both cells in all times of exposure	Fukushima et al. (2013)
- Halloysite clay nanotubes (HNT)	Unmodified	Caco-2/HT29-MTX cells in co-culture	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 H-tetrazolium-5-carboxanilide (XXT) assay, LDH release and Lucifer yellow permeability assay	1, 10 and 100 μg/mL	6 h	No cytotoxic effects were observed in any assay performed	Lai et al. (2013)

- CNa^+ - C30B	Unmodified Quaternary ammonium salt (MT2EtOH)	HepG2 cell line	Protein content, neutral red uptake, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) reduction assay	0-62.5 μg/mL 0-500 μg/mL	24, 48 h	Only C30B showed cytotoxic effects (EC_{50}=88 μg/mL)	Maisanaba et al. (2013)
- 6-MP+ Mt-PLA nanocomposite	6-Mercaptopurine (6-MP) encapsulated with Mt	IMR32 cell line	Tryan blue dye exclusion test and MTT assay	10 ppm/test composite	24 h	Cell viability was greater after exposure to the nanocomposite containing the drug (6-MP)	Kevadiya et al. (2013)
-C20A -Clay1 -Clay2	Quaternary ammonium salt (2M2HT) Hexadecyltrimethylammonium bromide (HDTA) HDTA+Acetylcoline (ACO)	Caco-2 and HepG2 cell lines	Protein content, MTS reduction assay	0-62.5 μg/mL 0-8 μg/mL 0-125 μg/mL	24, 48 h	Only Clay2 induced cytotoxicity in both cell lines, being more sensitive Caco-2 than HepG2 (EC_{50}=34 μg/mL and EC_{50}=88 μg/mL, respectively)	Houtman et al. (2014)
- CNa^+ - C30B	Unmodified Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	Protein content, neutral red uptake, MTS reduction assay	0-125 μg/mL 0-250 μg/mL	24, 48 h	Only C30B showed cytotoxic effects (EC_{50}=40 μg/mL)	Maisanaba et al. (2014a)
-Clay1 -Clay2	Hexadecyltrimethylammonium bromide (HDTA) HDTA+Acetylcoline (ACO)	Caco-2 and HepG2 cell lines	Neutral red uptake	0-8 μg/mL 0-125 μg/mL	24, 48 h	No significant effects were observed, except for a decrease in NR uptake in HepG2 cells exposed to 8 μg/mL Clay1	Jorda-Beneyto et al. (2014)
-PLA-Clay1 extract -PLA-Clay2 extract	HDTA+Mt+ PLA HDTA+ACO+Mt+PLA	Caco-2 and HepG2 cell lines	Protein content, neutral red uptake, MTS reduction assay	0-100% of extract	24, 48 h	No cytotoxic effects were recorded in both cell lines at any concentration and exposure time	Maisanaba et al. (2014b)
- CAP+ Mt-PLA nanocomposite	Capecitabine (CAP) encapsulated with Mt	IMR32 cell line	Tryan blue dye exclusion test and MTT assay	10 ppm/test composite	24 h	Cell viability was greater when cells were exposed to the nanocomposite containing the drug (CAP) than when exposing cells only to CAP	Kevadiya et al. (2014)
- CNa^+ - C30B - Clay1 - Clay2	Unmodified Quaternary ammonium salt (MT2EtOH) HDTA HDTA+ACO	HUVEC cell line	Protein content, neutral red uptake, MTS reduction assay	0-125 μg/mL 0-250 μg/mL 0-8 μg/mL 0-125 μg/mL	24, 48 h	CNa+ and Clay1 showed in general no significant changes in any of the endpoints assayed after 24 and 48 h of exposure. C30B and Clay2 induced cytotoxic effects (EC_{50}= 21.4 ± 11.4 μg/mL and EC_{50}=55.4 ± 6.1 μg/mL, respectively) both at MTS after 24 h of exposure	Maisanaba et al. (2014c)
- Unmodified MT (MMTdell and MMTdells)	Dimethyl dihydrogenated tallow ammonium	Ramos, A-549, HCT-116, SK MEL 28 and HepG2 cell lines	Alarm Blue assay	Serial dilutions from 10^{-2} to 10^2 μg/mL	72 h	The nanoclays modified with dimethyl benzyl hydrogenated tallow ammonium showed higher toxicity than those modified with the dimethyl dihydrogenated tallow ammonium	Janer et al. (2014)
- Modified MT (MMTdell 43B, MMTdell 67G, MMTdell 72T, MMTdell 43Bs, MMTdell 67Gs and MMTdell 72Ts)	Dimethyl benzyl hydrogenated tallow ammonium						

Cell lines: A-549: human alveolar adenocarcinoma cell line; Caco-2: colorectal adenocarcinoma cell line; CCD-986sk: human skin fibroblast; CHO: Chinese hamster ovary cell line; HCT-116: human colorectal carcinoma cell line; HEK 293: human embryonic kidney cell line; HeLa: human epithelial adenocarcinoma cell line; HepG2: liver hepatocellular carcinoma cell line; HMy2.CIR: human B lymphoblast cell line; HT-29: human colon epithelial carcinoma cell line; HT29-MTX: human colon adenocarcinoma cells treated with methotrexate; HUVEC: human endothelial cell line; IMR32: human neuroblastoma cell line; IMR90: human lung fibroblast cell line; INT-407: human embryonic intestine cell line; L929: murine fibroblast cell line; MRC-5: human lung fibroblast cell line; MCF-7: human breast cancer cell line; NIH3T3: mouse fibroblast cell line; N1E-115: neuroblastoma cell line; SK MEL 28: human melanoma cell line; Ramos: human Burkitt's lymphoma cell line; ROC-1: oligodendroglial cell line.

(Meibian et al., 2010). In fact, it has been reported that different modifiers have different effects; hence, Mt delite modified with dimethyl benzyl hydrogenated tallow ammonium showed higher toxicity than those modified with dimethyl dihydrogenated tallow ammonium in five cell lines (Janer et al., 2014). This finding was corroborated by analysing the cytotoxic effects of the modifiers in the same cell lines. Studies evaluating the toxicity of quaternary ammonium compounds potentially used as clay modifiers are scarce. Thus, Jodynis-Liebert et al. (2010) obtained a mean inhibitory concentration for didecyldimethylammonium saccharinate among 1.44 and 5.47 μM for the MTT assay in six human cell lines. Moreover, native Mt (CNa^+) showed lower toxicity than the modified Mt (C30B) in HepG-2 and Caco-2 cells (Maisanaba et al., 2013, 2014a). Additionally, the same cell lines showed the different cytotoxic profiles of the three organo-modified clay minerals, evidencing that the type of clay mineral, the concentration range and the origin of the cell line play an important role in the observed toxicity (Houtman et al., 2014; Jordá-Beneyto et al., 2014). However, the modification of Mt was also demonstrated to decrease the toxicity of clay minerals. For instance, the modification of Mt with oligo (styrene-co-acrylonitrile) resulted in a less toxic material compared with the unmodified Mt (Liu et al., 2011). In addition, the modification of the organoclay could result in a non-toxic material, which is the main objective of the synthesis of these materials intended for human contact. Han et al. (2011) reported no significant membrane damage and change in cell viability in four cell types exposed to magnesium and calcium organophyllosilicates.

The nature of the clay mineral plays an important role in the observed toxicity. In this regard, Murphy et al. (1993a) reported the following toxicity scale in HUVE cells after 24 h of incubation with 0.1 mg/mL clay minerals: Mt > bentonite = kaolinite ⩾ erionite. In addition to the above-mentioned work, most studies have evaluated the toxicity of Mt and bentonite. To the best of our knowledge, the toxicity of kaolinite has only been studied by Gao et al. (2000), who compared the results with another silicate, namely quartz. Both compounds induced cytotoxic effects on rat pulmonary alveolar macrophage cells starting on the first day of exposure, although pretreatment with surfactant delayed the toxicity.

In addition to the platelet clays, tubular nanoclays, another physical structure of clay minerals, have been assayed. Verma et al. (2012) compared the toxicity of both structures in the lung epithelial cells A549 and showed that the platelet-structured nanoclays were more cytotoxic than the tubular types. It is important to note that the structure was not the only difference among them because the nature of the clay mineral also differed. In this sense, platelet clays were mainly bentonites, whereas tubular nanoclays were halloysites. In this regard, the toxicity of halloysite nanotubes (HNT) has also been evaluated in a co-culture of intestinal cells exposed to 0–100 μg/mL HNT, which resulted in no cytotoxic effect (Lai et al., 2013). However, unmodified and functionalized HNTs showed cytotoxic effects on HeLa and MCF-7 cells at concentrations higher than 75 μg/mL (Vergaro et al., 2010). No influence on the functionalization of the HNT was observed. Liu et al. (2012) assessed the safety of HNT incorporated into chitosan to form bionanocomposite films. The Chitosan/HNTs nanocomposite showed similar cytocompatibility to the chitosan nanocomposite without the clay nanotube. Despite the latter work, very few studies have been conducted to date in composites containing clay minerals. Only Kevadiya et al. (2013, 2014) have reported the interesting potential of clay-based composites as reservoirs of cytotoxic drugs because they have been proven to reduce the toxic effects of the drugs and to also play an important role in the delivery proficiency.

Among the most frequently used endpoints in cellular toxicity testing are those based on changes in cell morphology (Borenfreund and Borrero, 1984). The most remarkable morphological features altered by clay minerals are listed in Table 4. The morphological changes induced by different clay minerals have been studied in HUVE cells, which were markedly changed after 24 h of exposure to 0.1 mg/mL Mt, bentonite and kaolinite, including cell lysis (Murphy et al., 1993a). The lysis was greater in the case of Mt compared with bentonite and kaolinite, with only limited lysis observed after exposure to erionite. This finding agreed with the cytotoxicity assay results. In the same manner, in shorter exposure times, Mt and bentonite were also able to cause complete cell lysis in the neuronal cultures within 60 min (Murphy et al., 1993b). Similarly, HepG2 cells exposed for 24 h to 50 and 1000 μg/mL CNa^+ and C93A exhibited a dose-dependent cell death induction (Lordan et al., 2011). Moreover, C30B induced morphological changes in HepG2 and Caco-2 cells after 24 and 48 h of exposure, undergoing ultrastructural features characteristic of cell impairment, such as damage to the mitochondria, nucleus and endomembrane systems (Maisanaba et al., 2013, 2014a). In contrast, no cell organelles were affected in the lung fibroblast cell line IMR90 exposed to 10 μg/cm^2 Mt for 24 h (Geh et al., 2006).

The degeneration of the mitochondria reported in HepG2 and Caco-2 cells exposed to C30B is in agreement with the changes observed in the MTT, MTS, WST-1 and XXT (different tetrazolium salts) assays in many cell types exposed to unmodified and functionalized Mt and HNT (Baek et al., 2012; Houtman et al., 2014; Lai et al., 2013; Li et al., 2010; Liu et al., 2011; Lordan et al., 2011; Maisanaba et al., 2013, 2014a,b,c; Vergaro et al., 2010). Additionally, the presence of fat droplets and nuclear lipid inclusions in HepG2 and Caco-2 cells exposed to C30B revealed an alteration in the lipid metabolism, which is consistent with the release of fatty acids reported by Murphy et al. (1993a).

In addition to the studies on clay minerals, the biocompatibility of bio-nanocomposites containing these materials has also been assessed by analysing the attachment and spreading of L-929 cells (Zia et al., 2011). The cells showed higher attachment and growth on bio-nanocomposites without Mt than those containing the nanoclay at a concentration of 1%, 2%, 4% and 8%. The staining results also showed that an increased in the concentration of Mt has adverse effects on the biocompatibility of the samples. In contrast, Liu et al. (2012) examined the cytocompatibility of chitosan/HNTs nanocomposites films, evidencing a positive response in NIH3T3 cells even at the highest content of HNTs (10%).

When all data above are considered, it is evidenced that *in vitro* toxicological research on clays is of high interest nowadays. However, reports dealing with nanocomposites containing clays are scarcer. Different clays have their own cytotoxic profile with dependence on the experimental conditions (type of clay, modifier, cell line, concentrations used, *etc.*). But they have been shown to be able to induce deleterious effects on cells, and this deserves to be investigated, taking into account their multiple applications.

2.2. Toxicity mechanisms

In vitro toxicological assessments are useful for obtaining mechanism-derived information. Cells respond rapidly to toxic stress by altering, for example, different basal biomarkers, such as the generation of reactive oxygen species (ROS) and glutathione content (GSH), and making alterations to several organelles, such as the mitochondria and lysosomes. Taking this into account, important questions are how the studied materials, in this case, clay minerals, and derived nanocomposites are able to alter these parameters and how these alterations can affect the metabolic rates, cell growth and gene transcription (Eisenbrand et al., 2002).

The interaction, influence and potential toxicity of materials with proteins and cells are an essential focus in assessing and

Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

Table 4
Morphological changes evaluation of unmodified/modified clays and derived nanocomposites.

Material tested	Modifier	Experimental model	Concentration range	Exposure time	Main results	Reference
- Mt and bentonite	Unmodified	HUVE cell line	0.1 mg/mL	24 h	All clays induced morphological changes, including lysis of the cells. The toxicity observed was: Mt > bentonite ≈ kaolinite»erionite	Murphy et al. (1993a)
- Mt, bentonite and erionite	Unmodified	Culture of primary murine spinal cord neurons and differentiated N1E-115 cell line	0.1 mg/mL	5, 15, 60 min and 18 h	Mt and bentonite caused complete cell lysis in the neuronal cultures within 60 min after exposure. None of the clays appeared to be cytotoxic to the differentiated NlE-115 cells even at 18 h	Murphy et al. (1993b)
- Native bentonite - Modified bentonites	Unmodified α-Quartz + chemical modifications (alkalin, acid and organic)	IMR90 cell line	10 μg/cm^2	24 h	No organelles were affected in IMR90 cells exposed to clays; despite Mt crystal was found intracellularly	Geh et al. (2006)
- CNa^+ - C93A	Unmodified Ternary ammonium salt (M2HT)	HepG2 cell line	50 and 1000 μg/mL	24 h	Cell death was observed after exposure to nanoclays	Lordan et al. (2011)
- Polyurethane bio-nanocomposites	4,4'- diphenylmethane diisocyanate	L-929 cell line	PUBNC1: without nanoclay, PUBNC2: 1.0%, PUBNC3: 2.0%, PUBNC4: 4.0% and PUBNC5: 8.0% nanoclay	48 h	No cytotoxicy was observed in cells exposed to the nanocomposites without nanoclay and that containing only 1% nanoclay. However, those with higher contents of bentonite nanoclay showed some toxic and incompatible behaviour	Zia et al. (2011)
- Nanocomposite of halloysite clay nanotubes (HNT) incorporated into chitosan	Unmodified	NIH3T3 cell line	Chitosan/HNTs with 5% and 10% HNTs	1, 3, and 7 days	Both chitosan and chitosan/HNTs nanocomposite films are cytocompatibility even when the loading of HNTs is 10%	Liu et al. (2012)
- C30B	Quaternary ammonium salt (MT2EtOH)	HepG2 cell line	0–88 μg/mL	24 and 48 h	Mitochondrial degeneration, dilated endomembrane systems, heterophagosomes formation, fat droplets appearance and presence of nuclear lipid inclusions were oberved	Maisanaba et al. (2013)
- C30B	Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	0–40 μg/mL	24 and 48 h	Dilated cisternae edge in the Golgi apparatus and nucleolar segregation was observed	Maisanaba et al. (2014a)

Caco-2: colorectal adenocarcinoma cell line; HepG2: liver hepatocellular carcinoma cell line; HUVE: human umbilical vein endothelial cell line; IMR90: human lung fibroblast cell line; L-929: fibroblast cell line; NIH3T3: mouse fibroblast cell line; and N1E-115: neuroblastoma cell line.

understanding the material compatibility versus toxicity (Jones and Grainger, 2009). The cell-material reactions of interest include cellular uptake, membrane integrity perturbations, or alteration in the viability or vital organelles. Thus, endpoints such as the generation of ROS and other parameters related with the cellular stress response (GSH, superoxide dismutase activity (SOD), lipid peroxidation (LPO), *etc.*), DNA damage, inflammation response and cell necrosis and apoptosis provide valuable information for understanding the toxic response.

Below, a description of the main studies that focused on the toxicity mechanisms in different cell lines exposed to clay minerals and derived nanocomposites is presented. Moreover, the similarities and differences in the results due to different experimental models and exposures will hopefully be identified.

2.2.1. Oxidative stress generation

Increases in the intracellular level of ROS represent a potential toxic mechanism, which if not counteracted will lead to other disorders, such as membrane dysfunction, LPO, which means a higher malondialdehyde level (MDA) in the cell membrane, DNA damage and a drastic inactivation of proteins. To prevent damage to cellular components, there are numerous enzymatic antioxidant defences designed to scavenge ROS in the cell. Examples of these enzymes are the SOD, catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Puerto et al., 2009, 2010). Activity measurements of this group of antioxidant enzymes may provide a marker of oxidative stress because they are able to be completed with the study of other parameters, such as the GSH, MDA and carbonyl levels (Puerto et al., 2014).

Several researchers have studied the effects related with ROS generation and other disorders in several target cell lines exposed to different unmodified and modified clay minerals and derived micro/nanocomposites (Baek et al., 2012; Governa et al., 1995; Hansen and Mossman, 1987; Houtman et al., 2014; Kevadiya et al., 2013, 2014; Lordan et al., 2011; Maisanaba et al., 2013, 2014a; Meibian et al., 2010; Sharma et al., 2010) (Table 5).

Contradictory results were observed when various cell lines from digestive origin, such as HepG2, Caco-2 and INT-407, were exposed to unmodified Mt, CNa^+. Baek et al. (2012) showed that the exposure of the INT-407 cell line for 72 h to the lowest concentration of Mt tested resulted in ROS generation. However, significant differences with respect to the control were only observed with the highest concentration assayed, 1000 μg/mL, after 24 h. Similarly, Lordan et al. (2011) also evidenced ROS generation in HepG2 cells exposed to CNa^+ starting with the lower concentrations (50–1000 μg/mL) and at all times of exposure (4, 16 and 24 h). Nevertheless, other researchers did not observe any effects in ROS generation in Caco-2 cells exposed to the unmodified clay at the concentrations (the highest was 226 μg/mL) and times assayed (Sharma et al., 2010). The stress response generated depends on the cell lines exposed and the concentration ranges assayed.

Lordan et al. (2011) also evaluated the possible ROS generation in the HepG2 cell line exposed to C93A, a modified Mt with a ternary ammonium salt. The results obtained were similar to those obtained after exposure to CNa^+, but the effect of C93A on intracellular ROS production was less prominent because the increased levels observed were not as substantial as those obtained with the unmodified clay and were not consistent over 24 h of exposure. Sharma et al. (2010) observed that C30B, a quaternary ammonium salt-modified clay, did not induce ROS production at the conditions assayed in Caco-2 cells. In contrast, our research group (Maisanaba et al., 2013; 2014a) also evaluated the stress response by analysing the ROS and GSH content of HepG2 and Caco-2 cells exposed to C30B. In our case, when HepG2 cells were exposed to the modified clay, no significant alteration in ROS production was observed at all concentrations (22, 44 and 88 μg/mL) and times of exposure (24 and 48 h). In contrast, a concentration-dependent decrease in the GSH content was obtained at the highest concentration tested and at both times of exposure. According to the results obtained by Maisanaba et al. (2013), Caco-2 cells exposed to C30B experienced a significant decrease in the GSH content at 40 μg/mL after 48 h. Moreover, an alteration in ROS generation was also observed at 40 μg/mL and at both times of exposure (24 and 48 h). The production of ROS may be related to the cell damage induced by C30B at this concentration, as was also corroborated by an aforementioned morphological study (Maisanaba et al., 2014a).

In contrast, Houtman et al. (2014) also evaluated the stress response in HepG2 and Caco-2 cell lines exposed to the other novel modified clay, Clay2, in the presence of the other quaternary ammonium salt modifier, HDTA+ACO. These researchers only observed an alteration in the GSH content in HepG2 cells exposed to the modified clay, and significantly differences were obtained at all concentrations tested (22, 44 and 88 μg/mL) and both times of exposure (24 and 48 h).

In addition to Mt, studies with other clay minerals with a similar structure have been published in the scientific literature. For example, Meibian et al. (2010) studied the ROS generation, SOD activity and MDA levels of a lymphoblast cell line exposed to two bentonites, an unmodified or native bentonite (BPN) and a modified or active bentonite (BPA) activated with H_2SO_4. In this study, the authors observed that the highest oxidative response was shown after exposure to BPA at all of the conditions tested.

Little is known about *in vitro* stress assays of nanocomposites obtained from clays and polymeric matrices. However, some studies of microcomposite materials used for different biological applications have been performed. Kevadiya et al. (2013) evaluated the stress effects of 6-mercaptopurine (an antineoplastic drug)-Mt-poly (L-lactide) acid (PLA) microcomposite (6-MPMtPLA) in the neuroblastoma cell line IMR32. The MDA levels, SOD activity, protein carbonyl levels and GSH content were evaluated, and the results showed that all of the endpoints in the assayed cells exposed to the test composite (10 ppm) did not show significant differences with respect to the control group. Specifically, an increase in the protein carbonyl groups was expressed in pristine 6-MP-treated cells, whereas 6-MP-Mt and microcomposite spheres (MPs) proved to be somewhat less toxic in this parameter. Intracellular glutathione estimation also revealed similar results as 6-MPMtPLA and MPs, *i.e.*, it was less damaging than the pristine antineoplastic drug. Similar results were obtained in another study in which the IMR32 cell line was exposed to capecitabine (CAP) (other antineoplastic drug) encapsulated in Na^+-Mt and further compounded with poly (L-lactide) acid. In all cases, cells treated with CAP-Mt and MPs exhibited behaviour similar to that of the control group, showing lower oxidative responses compared with the pristine CAP cell group (Kevadiya et al., 2014). In this sense, the encapsulation of the antineoplastic drugs into the clays has ameliorated the toxicity.

2.2.2. Genotoxicity assays

Due to the variety of mechanisms leading to xenobiotic-induced DNA damage and the range of mutagenic events that may occur as a result, a battery of testing systems is required for the establishment of the genotoxic potential of a substance under investigation (Doak et al., 2012). At present, *in vitro* mutagenicity assays are included among the core set of toxicity tests for the safety assessment required by European legislation to authorize different substances. The most frequently used genotoxicity assays are the Comet assay, Ames test and micronucleus assay (Maisanaba et al., 2013, 2014a,b,c).

In this regard, the genotoxicity evaluation of clays and derived nanocomposites used in or destined for the food industry is of

Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

Table 5
Oxidative stress evaluation of unmodified/modified clays and derived micro/nanocomposites.

Material tested	Modifier	Experimental model	Assays performed	Concentration range	Main results	Reference
- Sepiolite	Unmodified	Hamster and rat alveolar macrophagues	Reduction of cytocrome *C* (SOD)	5 μg/cm^2 dish and 25 μg/cm^2 dish in each cell line, respectively	The clay mineral caused a significant increase in the release of O_2^- in both cell lines	Hansen and Mossman (1987)
- Sepiolite - Kaolinite	Unmodified (both of them)	PMN cell line AM cell line	ROS generation	Not specified	Sepiolite was unreactive in ROS production while a high ROS generation was observed after kaolinite exposure	Governa et al. (1995)
- Native bentonite (BPN) - Active bentonite(BPA)	Unmodified H_2SO_4	HMy2.CIR cell line	ROS generation, SOD activity, MDA levels	0–240 μg/mL	The highest oxidative response was presented by BPA exposure	Meibian et al. (2010)
- CNa^+ - C30B (unfiltered and filtered)	Unmodified Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	ROS generation	0–226 μg/mL	ROS production was not observed	Sharma et al. (2010)
- CNa^+ - C93A	Unmodified Ternary ammonium salt (M2HT)	HepG2 cell line	ROS generation	0–1000 μg/mL	ROS production was observed with both clays	Lordan et al. (2011)
- Mt	Unmodified	INT-407 cell line	ROS generation	0–1000 μg/mL	ROS production was observed	Baek et al. (2012)
- 6-MP+Mt-PLA microcomposite	6-Mercaptopurine (6-MP) encapsulated with Mt	IMR32 cell line	MDA levels, SOD activity, protein carbonyl levels, GSH content	10 ppm/test composite	In all cases cells exposed to test composites showed lower oxidative responses than the other groups assayed	Kevadiya et al. (2013)
- C30B	Quaternary ammonium salt (MT2EtOH)	HepG2 cell line	ROS generation, GSH content	0–88 μg/mL	No significant alteration in ROS generation was observed but a decrease of GSH content was showed	Maisanaba et al. (2013)
- Clay2	Hexadecyltrimethylammonium bromide (HDTA)+Acetylcoline (ACO)	Caco-2 and HepG2 cell lines	ROS generation, GSH content	0–34 μg/mL and 0–88 μg/mL	Both cell lines exposed to Clay2 did not present ROS generation. HepG2 showed a significant decreased in GSH content	Houtman et al. (2014)
- CAP+ Mt-PLA microcomposite	Capecitabine (CAP) encapsulated with Mt	IMR32 cell line	MDA levels, SOD activity, Protein Carbonyl levels, GSH content	10 ppm/test composite	In all endpoints, cells exposed to test composites showed lower oxidative responses than the other groups assayed	Kevadiya et al. (2014)
- C30B	Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	ROS generation, GSH content	0–40 μg/mL	Increased ROS and decreased GSH content at the highest concentration	Maisanaba et al. (2014a)

Cell lines: PMN: human polymorphonuclear leucocytes; AM: bovine alveolar macrophagues; HMy2.CIR: human B lymphoblast cell line; Caco-2: colorectal adenocarcinoma cell line; HepG2: liver hepatocellular carcinoma cell line; INT-407: human normal intestinal cell line: and IMR32: human neuroblastoma cell line.

great interest. In previous opinions (EFSA, 2011b and 2012), the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has assessed the safety of bentonite and concluded that "montmorillonites are not genotoxic based on a series of tests" (EFSA, 2013), but different results have been obtained to date.

An important number of studies related to the possible carcinogenic and mutagenic effects of clay minerals in different cell lines are available in the scientific literature (Table 6) (Denizeau et al., 1985; Gao et al., 2000; Houtman et al., 2014; Li et al., 2010; Maisanaba et al., 2013; Maisanaba et al., 2014a,b,c; Meibian et al., 2011; Sharma et al., 2010).

Several research groups have evaluated the DNA damage induced by unmodified Mt and CNa^+ through different methods and experimental models, and all of them agree that Mt does not exhibit genotoxic or mutagenic potential (Li et al.,2010; Maisanaba et al., 2014c; Sharma et al., 2010). However, different results were obtained in the case of modified clays. Maisanaba et al. (2013) evaluated the DNA damage using the comet assay in HepG2 cells exposed to C30B, evidencing DNA strand breaks at the highest concentration assayed (88 μg/mL) after 48 h of exposure. However, a similar experiment in another cell line, Caco-2, resulted in no DNA damage (Maisanaba et al., 2014a). In contrast, Sharma et al. (2010) also studied the exposure to C30B in Caco-2 cells, and genotoxic effects in a concentration-related manner were observed. In this case, the differences between the results could be directly related to the concentration assayed because Sharma et al. (2010) used higher concentrations in comparison to Maisanaba et al. (2014a). Moreover, the genotoxic potential of two novel modified clays, Clay1 and Clay2, has also been evaluated by the comet assay and compared with that of another modified clay, C20A. In this case, only DNA damage was observed in cells (Caco-2 and HepG2) exposed to Clay2 at the highest concentrations tested (34 and 44-88 μg/mL, respectively) (Houtman et al., 2014).

Other researchers have studied similar structural clays, such as bentonite and kaolinite. Gao et al. (2000) evaluated the genotoxic effects in rat pulmonary alveolar macrophages exposed to unmodified quartz and kaolinite and their modifications with dipalmitoylphosphatidylcholine (DPPC) by a single-cell gel electrophoresis assay to detect the DNA damage induced. The results show that quartz and kaolin, regardless of whether it is treated, causes genotoxic effects, but DPPC-treated kaolin showed no statistically significant activity until day 5 at the highest concentration (40 μg/mL), whereas the other samples induce DNA damage at lower exposure times.

Apart from the comet assay, other mutagenicity trials are performed to evaluate different alterations that could occur in the genetic material. The Ames test, according to the OECD (1997) guideline for the testing of chemicals 471, is the bacterial reverse mutation test used to determine the mutagenicity of exogenous substances. The test identifies mutagenic compounds as those capable of reverting point mutations in histidine or tryptophan biosynthesis genes in *Salmonella typhimurium* or *Escherichia coli*, respectively, restoring the ability of the bacteria to generate these essential amino acids. Usually, a combination of 5 *S. typhimurium* strains or 4 *S. typhimurium* strains plus 1 or 2 *E. coli* strains are required to detect a range of base substitution or frameshift events. The ease and cost effectiveness of the test system make it widely used in the safety analysis of chemical substances. It is an essential test within the current battery of assays required for genotoxicity evaluation (Doak et al., 2012).

According to the OECD (2010) guideline 487, the *in vitro* micronucleus assay (MNvit) is a test that rapidly determines the frequency of gross chromosomal damage induced by a test agent. It has gained popularity as the test of choice over the chromosome aberration assay because it is substantially quicker to perform and easier to analyse and readily detects aneugens and clastogens (which the chromosome aberration assay cannot do unless it is specifically modified). Thus, the MNvit is now recommended as one of the *in vitro* test systems to characterize the genotoxicity of chemical and pharmaceutical agents (Kirkland et al., 2011).

Sharma et al. (2010) studied the mutagenic potential of CNa^+ and C30B in both filtered and unfiltered suspensions. In any case, there were no indications of mutagenic activity in the two strains, TA98 and TA100, assayed either with or without metabolic activation up to the highest soluble concentration (141 μg/mL). However, Maisanaba et al. (2014c) also evaluated the mutagenic potential of CNa^+ and C30B by the Ames test, obtaining positive results only in the case of the modified clay C30B in the presence of the S9 fraction at four of the five concentrations assayed (31.25–250 μg/mL). In the same work, other quaternary ammonium salt-modified clays, Clay1 and Clay2, showed a different mutagenic profile, and only Clay1 showed positive results at the four highest concentrations (1–8 μg/mL) with S9. The presence of mutagenicity may be related to the modifier selected, the range of concentrations assayed or the strains used in the test.

In addition to the mutagenic evaluation of CNa^+, Taylor et al. (2014) also investigated the mutagenicity potential of halloysite. The most remarkable results from the Ames mutagenicity test showed a slight but reproducibly elevated growth at all concentrations of CNa^+ assayed. The authors associated this finding to the presence of a beneficial trace mineral component in CNa^+. However, in the case of halloysite, a diminished growth in the bacterial strains suggested toxicity rather than a mutagenic effect.

Meibian et al. (2011) also evaluated the possible genotoxic alteration in the MHy2.CIR cell line exposed to BPN and BPA through the comet and micronucleus assays. These results correlate well with those from the afore-mentioned oxidative stress studies.

Reports on the mutagenicity of nanocomposite materials are even scarcer. Maisanaba et al. (2014b) analysed the mutagenic potential of PLA-Clay1 and PLA-Clay2 extracts by the Ames test. These were obtained from a nanocomposite formed by Clay1 + PLA and Clay2 + PLA, respectively, and did not present mutagenic potential in any case. Also, genotoxicty and mutagenicity studies on potential modifiers are lacking and the results available shown both positive and negative effects (Dmochowska et al., 2011; Grabinska-Sota, 2011).

Anew, different results dependent on the clays assayed, the present modifier (if any) and the concentration range tested are presented in this section.

2.2.3. Inflammation responses

Among the biomarkers that are most frequently assayed in relation to the inflammation process is the cytokine release, mainly interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (Lopresti et al., 2014).

Inflammation studies related with modified clays used in the food industry are scarce. The Caco-2 and HepG2 cell lines did not experiment an increase in IL-6 release after exposure to the modified clays, Clay2 and C30B (Houtman et al., 2014; Maisanaba et al., 2013). However, Elmore (2003) reported that different clays, including Mt, induced cytotoxicity in several macrophage-type cell lines and have haemolytic activity toward the red blood cells of different species. Additionally, it has been reported that Mt clay may promote infection by a direct cytotoxic effect on neutrophils, making them unavailable for bacterial phagocytosis (Dougherty et al., 1985).

2.2.4. Cell death

Different markers are used to determine the cell death mechanism after exposure to a toxic substance. Among the most used are flow cytometry and caspase activities (Napierska et al., 2009;

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Table 6
Genotoxicity of unmodified/modified clays and derived nanocomposites.

Material tested	Modifier	Experimental model	Assays performed	Concentration range	Main results	Reference
- Sepiolite	Unmodified	Rat hepatocytes	Unscheduled DNA synthesis (UDS)	1 and 10 μg/mL	Sepiolite did not lead to significant UDS-induction	Denizeau et al. (1985)
- Quartz - Kaolin (both untreated and treated)	Dipalmitoyl phosphatidylcoline (DPPC)	Rat pulmonary alveolar macrophagues cell line	Single-cell gel electrophoresis assay for DNA damage	0–40 μg/mL	Genotoxic effects were observed in all cases	Gao et al. (2000)
- NSP	Unmodified	*Salmonella typhimurium* strains (TA98,TA100, TA1535, TA1537, TA102)	Ames test	0–1000 μg/plate	No significant genotoxic effects were observed in any assay	Li et al. (2010)
		Rat peripheral-blood cell line	Micronucleus assay	0–500 mg/kg bw		
		CHO cell line	Comet assay	0–1000 μg/mL		
- CNa^+	Unmodified	*Salmonella typhimurium* strains (TA98,TA100)	Ames test	0–14.1 μg/plate	Genotoxic effects were observed by Comet assay in Caco-2 cells exposed to C30B	Sharma et al. (2010)
- C30B (unfiltered and filtered)	Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	Comet assay	0–170 μg/mL (unfiltered)/0–226 μg/mL (filtered)		
- Native bentonite (BPN) - Active bentonite(BPA)	Unmodified H_2SO_4	HMy2.CIR cell line	Comet assay Micronucleus assay	0–240 μg/mL	The highest genotoxic effects were observed after BPA exposure	Meibian et al. (2011)
- Bentonite	Unmodified	*Salmonella typhimurium* strains (TA97, TA102)	Ames test	0–10 mg/plate	No mutagenic activity was observed	EFSA (2013)
- C30B	Quaternary ammonium salt (MT2EtOH)	HepG2 cell line	Comet assay	0–88 μg/mL	DNA damage was observed in HepG2 exposed to C30B	Maisanaba et al. (2013)
- C20A - Clay1	Quaternary ammonium salt (2M2HT) Hexadecyltrimethylammonium bromide (HDTA)	Caco-2 and HepG2 cell lines	Comet assay	0–34 μg/mL and 0–88 μg/mL	Genotoxic effects were observed only in cells exposed to Clay2	Houtman et al. (2014)
- Clay2	HDTA+Acetylcoline (ACO)					
- C30B	Quaternary ammonium salt (MT2EtOH)	Caco-2 cell line	Comet assay	0–40 μg/mL	No DNA damage was observed	Maisanaba et al. (2014a)
- PLA-Clay1 extract - PLA-Clay2 extract	HDTA+Mt+ PLA HDTA+ACO+Mt+PLA	*Salmonella typhimurium* strains (TA97, TA98,TA100, TA102, TA104)	Ames test	0–100% of extract	No mutagenic potential was observed	Maisanaba et al. (2014b)
- CNa^+	Unmodified	*Salmonella typhimurium* strains (TA97, TA98,TA100, TA102, TA104)	Ames test	0–125 μg/mL	Mutagenic activity was observed for C30B and Clay1 exposure	Maisanaba et al. (2014c)
- C30B	Quaternary ammonium salt (MT2EtOH)			0–250 μg/mL		
- Clay1	HDTA			0–8 μg/mL		
- Clay2	HDTA+ACO			0–125 μg/mL		
- CNa^+	Unmodified	*Salmonella typhimurium* strains (TA1537, TA1538, TA102)	Ames test	0–0.01 g	A slight elevated growth was observed in CNa^+ exposure while a disminished growth was detected in Hallosyte exposure	Taylor et al. (2014)
- Hallosyte	Unmodified					

CHO: Chinese hamster ovary cell line; HMy2.CIR: human B lymphoblast cell line; Caco-2: colorectal adenocarcinoma cell line; and HepG2: liver hepatocellular carcinoma cell line.

Lordan et al., 2011)

Some studies related to the evaluation of cell death produced by exposure to clays can be found in the scientific literature (Table 7) (Geh et al., 2006; Janer et al., 2014; Lordan et al., 2011; Liu et al., 2011; Maisanaba et al., 2013; Meibian et al., 2010). Liu et al. (2011) studied the enzymatic activity of caspase 3 and the percentage of apoptotic/necrotic cells by flow cytometry in two different cell lines, NIH3T3 and HEK293, exposed to unmodified Mt and oligo(Styrene-Co-Acrylonitrile)-modified Mt (PSAN-Mt) at 1 g/L. The results of the two assays provided substantial evidence that less cell apoptosis was induced by PSA-Mt compared with Mt in both treated cells, although significant differences were observed with respect to the control in both clay exposures. Similar results were obtained by Janer et al. (2014), who evaluated the same parameters in the HepG2 and SKMEL28 cell lines exposed to Mt and other modified montmorillonites with quaternary ammonium modifiers. An increase in the caspase 3/7 activities were observed after 4.5 h only in the HepG2 cell line and in both cell lines at the highest time of exposure (48 h). Regarding the flow cytometry results, HepG2 exposed to 100 μg/mL large pristine nanoclays for 48 h showed a sevenfold increase in the percentage of cells stained with Annexin V. However, these results did not match those obtained by Lordan et al. (2011) because after 24 h, no increase in caspase 3/7 activity was detected in the cells treated with CNa+, the unmodified Mt, and C93A. Similarly, Maisanaba et al. (2013) did not detect any variation in caspase 3/7 activity in the HepG2 cell line exposed to C30B for 24 and 48 h.

Native and modified bentonites have also been evaluated by other research groups. Geh et al. (2006) used several techniques to show that the IMR90 cell line exposed to native or alkali-, acid- or organo-modified bentonites at a specific concentration (20 μg/mL) suffered necrosis and apoptosis as the cell death response. Meibian et al. (2010) also detected apoptotic cells after exposure to BPN and BPA in the HMy2.CIR cell line.

Regarding to the toxicity mechanisms of clays, cell death, genotoxicity, mutagenicity and oxidative stress have been widely investigated in the recent years, as it has been described above. Again, clays show specific toxicity profiles. On the other hand, the research on inmunomodulatory effects of clays is still scarce and it should be promoted as allergic disorders have a high prevalence nowadays.

2.3. Antimicrobial activity

In this era, when many bacteria have developed antibiotic resistance and may produce more harmful toxins as a defence mechanism (*e.g.*, Shiga-toxin), the investigation of natural alternative antibacterials is of paramount importance. Natural clays have been used for healing singe the earliest recorded history, but their medicinal properties have largely not been scientifically recognized (Williams et al., 2011). A variety of physical and/or chemical processes can provide antibacterial properties to clays. Physical bactericide can occur by surface attraction between clay minerals and bacteria, which can hamper the passive and active uptake of essential nutrients, disrupt cell envelopes or impair the efflux of metabolites (Ferris et al., 1987). The natural antibacterial clays studied do not kill by physical associations between the clay and bacterial cells (Williams and Haydel, 2010). Williams et al. (2011) described various studies verifying the antimicrobial potential against *E. coli* using clay minerals and Fe-rich phases. However, two works related to the Mt antibacterial action by nanohybrids or derived nanocomposites with polyurethane are available in the literature (Su et al., 2009; Wang et al., 2012a). Su et al. (2009) evaluated the potential against several bacterial strains (*Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyrogens, E. coli* and the methicillin- and oxacillin-resistant

Table 7
Cell death evaluation of unmodified/modified clays and derived nanocomposites.

Material tested	Modifier	Experimental model	Assays performed	Concentration range	Main results	Reference
- Native bentonite - Modified bentonites	Unmodified α-Quartz+chemical modifications (alkalin, acid and organic)	IMR90 cell line	Flow cytometry, gel electrophoresis	20 μg/mL	The bentonite samples induced necrosis as well as apoptotic cell death	Geh et al. (2006)
- Native bentonite (BPN) - Active bentonite (BPA)	Unmodified H_2SO_4	HMy2.CIR cell line	Flow cytometry	0–240 μg/mL	Bentonite particles induced apoptosis	Meibian et al. (2010)
- Mt - Oligo(styrene- Co- acrylonitrile) modified Mt (PSAN-Mt)	Unmodified Oligo(styrene-Co-acrylonitrile)	NIH3T3 cell line HEK293 cell line	Caspase 3 enzymatic activity, Flow cytometry	1 g/L	Caspase 3 activity and apoptosis were observed in both cases, but lower with PSAN-MMT	Liu et al. (2011)
- CNa^+ - C93A - C30B	Unmodified Ternary ammonium salt (M2HT) Quaternary ammonium salt (MT2EtOH)	HepG2 cell line HepG2 cell line HepG2 cell line	Caspase 3/activity Caspase 3/7 activity Caspase 3/7 activity, flow cytometry	0–1000 μg/mL 0–500 μg/mL 0–500 μg/mL	No increase in caspase 3/7 activity was detected The caspase 3/7 activity did not show any significant variation Significant differences were observed in both biomarkers assayed	Lordan et al. (2011) Maisanaba et al. (2013) Janer et al. (2014)
- Mt - Modified montmorillonites (nanoclays)	Unmodified Quaternary ammonium modifiers (dymethyl dehydrogenated tallow ammonium)	HepG2 cell line SKMEL28 cell line	Caspase 3/7 activity, flow cytometry	0–500 μg/mL		Janer et al. (2014)

IMR90: human fibroblast cell line; HMy2.CIR: human B lymphoblast cell line; NIH3T3: mouse embryonic fibroblast cell line; HEK293: human embryonic kidney cell line; HepG2: liver hepatocellular carcinoma cell line; and SKMEL28: human melanoma cell line.

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S. aureus) of nanohybrids synthesized via silver nitrate reduction in the presence of silicate clay, obtaining a higher potency against bacterial growth. The researchers proved that the Ag/silicate (7/93) ratio could inhibit the growth of dermal pathogens. Different antibacterial activity tests were carried out, and the results concluded that the nanohybrids are effective against a broad spectrum of bacteria, including the resistant bacterial strains, in both agar and solution. Furthermore, Wang et al. (2012a) demonstrated the antimicrobial activity of the exfoliated clay nanosilicate platelets (NSP), three types of surfactant-modified NSP (NSQa (cationic), NSQb (nonionic) and NSQc (anionic)), and the nanocomposites made from polyurethane (PU) and NSQs. NSQ modified by a cationic or anionic surfactant (NSQa or NSQc) displayed excellent antimicrobial activity against *S. aureus* and *E. coli* at a concentration of 1%. The addition of 1% NSQa in the PU matrix inhibited the growth of bacteria.

3. *In vivo* toxicological assays

The number of recent *in vivo* experimental toxicity studies with clay minerals in the scientific literature is low in comparison to the *in vitro* studies, and the results reported are variable because different clay minerals, exposure routes and concentrations have been used (Table 8). There are two previous reviews on the toxicity of clay minerals, and these were performed by Elmore (2003) and WHO (2005). Most of the studies included in those reviews were performed a long time ago, and an effort to avoid duplicates was made in the present work.

The exposure to clay minerals results in both toxic and non-toxic responses. Most of the previous studies show that clay minerals do not induce macro-toxic or histopathological effects after acute (Baek et al., 2012; Lee et al., 2005; Mascolo et al., 2004) or subchronic/chronic exposures (Afriyie-Gyawu et al., 2005; EFSA, 2013; Maisanaba et al., 2014d,e) in rodents. In fact, Baek et al. (2012) estimated that the median lethal dose (LD50) of Mt was >1000 mg/kg in mice. The absence of toxicity or very low toxicity was also reported by Wiles et al. (2004) in Sprague–Dawley rats exposed to Mt during pregnancy, with no significant differences between the animals and the embryos. However, Patterson and Staszak (1977) found developmental effects, with maternal anaemia and reduction in the birth weight of pups after exposure to 20% kaolin during gestation. However, the consumption of clays by wild animals in nature is common, and the practise of eating clay by wild animals is well documented (Slamova et al., 2011). Several hypotheses have been proposed to explain the geophagic behaviour: (1) detoxification of noxious or unpalatable compounds present in the diet, (2) alleviation of gastrointestinal upsets, such as diarrhoea, (3) supplementation of the body with minerals and (4) alleviation of hyperacidity in the digestive tract (Wilson, 2003). In fact, many reports are available on the ability of clay to reduce mycotoxicosis, metals, *etc*. in different animal models without inducing inherent toxicity, but this is beyond of the scope of this review.

To the best of our knowledge, in the last 10 years, the toxicity mechanisms of clay minerals has been scarcely investigated through *in vivo* methods. Regarding oxidative stress biomarkers, Kibanova et al. (2009) reported that nontronites induced higher LPO than hectorite in brain supernatant solutions exposed to up to 1000 ppm. Maisanaba et al. (2014e), however, did not find any alteration in the LPO, SOD, GPx and GST activities in the liver and kidney of rats exposed for 90 days to a 40 mg/kg bw/d Mt-modified clay (Clay1), although the CAT activity, gene expression and protein abundance were increased in the kidney. In relation to genotoxicity, EFSA (2013) referred to a study in which no chromosomal aberrations were detected in rats exposed to bentonite through diet for 15 days. Sharma et al. (2014) did not observe DNA strand-breaks in the colon, liver and kidney cells of Wistar rats exposed by gavage to 250–1000 mg/kg bw Closite®30B. These authors also reported the absence of inflammatory responses. This result agreed with those reported by Hsu et al. (2012), who exposed Sprague–Dawley rats to a subcutaneous injection of delaminated Mt particles with no induction of tissue inflammation. The International Agency for Research on Cancer (IARC) evaluated sepiolite and considered that it cannot be classified due to its carcinogenicity to humans (Group 3) based on limited evidence in experimental animals on the carcinogenicity of long sepiolite fibres (>5 μm) and inadequate evidence in experimental animals of the carcinogenicity of short sepiolite fibres (<5 μm) (IARC 1997). No carcinogenicity data on kaolinite or Mt were provided.

Some researchers have justified the absence of toxic effects because a systemic exposure was not demonstrated (Sharma et al., 2014). However, Baek et al. (2012) observed no toxicity even at 1000 mg/kg bw Mt but reported that the clay mineral could be absorbed into the body within 2 h without a specific organ for accumulation. To investigate the intestinal absorption of clay minerals, the content of the characteristic structural metals (mainly Al for kaolinites and smectites) is quantified. In this regard, contradictory results have been found in the scientific literature. Mascolo et al. (1999, 2004) observed a progressive increased accumulation of metals in the urine and tissues of rats in the following order: kidney > liver > heart > brain. Additionally, Reichardt et al. (2007) reported that kaolinite is dissociated in the lumen and that Al passes directly through the intestinal barrier. In contrast, Sharma et al. (2014) did not find an increase in the Al content in the liver and kidney of rats exposed by gavage to 1000 mg/kg bw Cloisite®30B. EFSA (2013) also considered that bentonite and sepiolite, similarly to other clays, are not absorbed to any measurable extent.

Taking into account that clays are modified to organoclays for their novel applications, the toxicity of the potential modifiers is also of interest. These investigations are scarce but Melin et al. (2014) found that quaternary ammonium compounds such as the mixture of alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride significantly impaired reproductive health in mice. On the other hand, a subchronic experiment in rats exposed by gavage to didecyldimethylammonium saccharinate revealed no treatment-related microscopic changes whereas the acute exposure to 2000 mg/kg bw resulted in death of all the animals (Jodynis-Liebert et al., 2010).

Regarding the toxicological evaluation of nanocomposites containing clays, only two studies have been identified. Hsu et al. (2012) reported that an Mt/chitosan nanocomposite showed higher *in vivo* compatibility compared with chitosan. Maisanaba et al. (2014f) exposed rats to the migration extract obtain from a PLA-Clay1 nanocomposite as a beverage and reported no toxic effects in the histopathological study nor in the oxidative stress biomarkers evaluated.

4. Environmental impact

4.1. Animal exposure

As was previously mentioned, clay minerals are currently used for numerous environmental applications due to their physicochemical properties. Some of these applications are the following: prevention of algal blooms, removal of organic/inorganic pollutants from soils and water, removal of pathogens, animal feeding, and treatment of feedlot waste (Bowman, 2003; Ferrario et al., 2000; Gillman, 2011; Lee and Tiwari, 2012). However, the presence of clay minerals on aquatic and terrestrial ecosystems may also

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Table 8
Toxicity assays with rodents exposed to unmodified/modified clay minerals and derived nanocomposites.

Material tested	Modifier	Experimental Model	Exposure	Concentration range	Main results	Reference
- Kaolin	Unmodified	Sprague-Dawley female rats	In the feed d37-68/69-95/96-117 prior fertilization and during gestation	0, 20% kaolin; iron-supplemented 20% kaolin	Rats fed kaolin diet exhibited maternal anaemia and reduction in the birth weight of the pups. These effects were not observed with the iron supplemented diet	Patterson & Staszak, (1977)
- Pharmaceutical clay (smectite) - Commercial herbalist clay (calcite, illite-mica, kaolinite, smectitic phases, quartz, feldspars, gypsum) - Natural clay (quartz, kaolinite, smectitic phases, sulphides and accessories)	Unmodified	Rats	Acute oral administration	0, 150, 350,450 mg/kg bw	The ingestion of clays causes a progressive increase of metals in the urine and presumably in the blood.	Mascolo et al. (1999)
- Pharmaceutical clay (Diosmectal©) - Commercial herbalist clay - natural clay	Unmodified	Male wistar rats	Intragastrically administered for 3 days using an orogastric tube. Trial of 6 days	450 mg/kg bw	No macrotoxic effects were detected. The trace element content in the organs is strictly related to the clay trace element content. The trace elemental distribution followed the order: kidney > liver > heart > brain	Mascolo et al. (2004)
- Calcium Mt clay (Novasil Plus) - Sodium Mt clay	Unmodified	Sprague-Dawley rats during pregnancy	In the feed and by gavage Until d16 of gestation	2% w/w	No significant differences for total implantations, resorptions or embryos. No significant differences in tissue weights, maternal body weight gain or feed intake. Only Rb levels were lowered significantly in treated animals but other tendencies were apparent	Wiles et al. (2004)
- Naturally occurring calcium Mt clay (NovaSil)	Unmodified	Sprague-Dawley rats	In the feed 28 weeks	0, 0.25, 0.5, 1.0, 2.0% w/w	No effects in either sex at the doses tested (total feed consumption, body weight, organ weight, histopathological changes, etc.) No dose-dependent and isolated differences in hematological parameters and clinical chemistry	Afriyie–Gyawu et al. (2005)
- Mt (particle size 92.2% > 8 μm)	Unmodified	Wistar rats	Oral and intravenous injection 72 h	A single dose 142.9 mg /kg bw oral exposure 14.29 mg/kg bw intravenous injection	Oral: increase in hemoglobin concentration, haematocrit and RBC count. Reduction in Na^{+}, Cl^{-} and Ca^{+2} Injection: no change in the haematological analysis. Increase in ALT, decrease in K^{+} No pathological alterations in any case	Lee et al. (2005)
- Kaolinite	Non-especified	Rats	Oral 28 days	Non-especified	Significant increase in the thickness of the villi, lengthening of the apical microvilli and increased mitochondrial replication. Stimulation of the absorption of long chain fatty acids. Kaolinite is dissociated in the lumen and Al passes directly through the intestinal barrier	Reichardt et al. (2007)
- Hectorite (SHCa-1) - Nontronites (NAu-1 & NAu-2)	Unmodified	Male wistar rats	Brain supernatant solutions were exposed to the clays	0-1000 ppm	Nontronites induced higher lipid peroxidation (LP) than hectorite. LP is clay-surface-controlled and dependent on clay structural composition	Kibanova et al. (2009)
- Uncoated and coated sepiolite nanoclay particulates - Quarz (SiO_2) - Ultrafine Titanium dioxide particles	Unmodified	Male Crl:CD(SD) Rats	Intratracheal instillation Analysis at 24 h, 1week, 5weeks, 3 moths postexposure	A single dose 1 mg/kg bw, 5 mg/kg bw	Sepiolite resulted in multinucleated giant cells at 1, 5 weeks and 3 months Increased BAL fluid lactate dehydrogenase values at 24 h Transient increases in cell labeling indices in the high dose group Lung inflammation. With increased time post installation, pulmonary changes became less severe	Warheit et al. (2010)
- Mt	Unmodified	Mice	Oral gavage	Single dose 5, 50, 300 and 1000 mg/kg bw	After 14 days post-administration no remarkable abnormalities were observed. LD50 > 1000 mg/kg bw	Baek et al. (2012)
- Delaminated Mt particles (DMtP) - Mt/chitosan (CS) nanocomposite	Unmodified	Sprague-Dawley rats	- Single subcutaneous injection.Sacrifice after 21 days	- 1 ml of 10, 10^2, 10^3, 10^4, 3 × 10^4 ppm DMtP solution - Film 10 mm x 10 mm,	- DMtP at low concentrations (≤ 100 ppm) did not provoke tissue reactions - Superior compatibility of DMtP/CS 10^3 ppm relative to CS. It was	Hsu et al. (2012)

- Bentonite nanoclay	Non available	Rats	- Subcutaneous insertion of a DMtP/CS film for 19 days Intragastric 28 days	≈0.1 mm thick Non available	degraded faster than CS in vivo Reduction of the relative weight of the liver, the activity of its conjugating enzymes, hyperproduction of colonic yeast microflora	Smirnova et al. (2012)
- Bentonite	Unmodified	Male rats	In the feed 15 days	0.5% w/w	No chromosomal aberrations No histopathological changes in liver and kidney	EFSA, 2013
- Sepiolite	Unmodified	Wistar rats	In the feed 22 weeks	1.5%	No effects on organ weights or gross pathology. No histopathological effects were observed in the liver, kidneys, stomach or intestine.	EFSA, 2013
- ToxFin®Dry (composed by bentonite and sepiolite)	Unmodified	Sprague-Dawley rats	In the diet 28 days	5000 mg/kg feed	Minor changes were seen in some parameters but it is concluded that there is no clear evidence of toxicity at 5000 mg/kg feed	EFSA, 2013
- C30B	Quaternary ammonium salt (MT2EtOH)	Wistar rats	Oral gavage Twice 24 h apart	250-1000 mg/kg bw	No induction of DNA strand-breaks in colon, liver and kidney cells. No increase in inflammatory cytokine markers in blood-plasma samples. No systemic exposure to clay particles.	Sharma et al. (2014)
- Clay1, Mt-modified clay	Hexadecyltrimethylammonium bromide (HDTA)	Male Wistar rats	With the diet 90 days	40 mg/kg bw/day	Non observable effects on the histopathology study, clinical biochemistry parameters and blood GSH/GSSG levels and IL-6 leakage	Maisanaba et al. (2014d)
- Clay1, Mt-modified clay	Hexadecyltrimethyl ammonium bromide (HDTA)	Male Wistar rats	With the diet 90 days	40 mg/kg bw/day	LPO, SOD, CAT, GPx, GST in liver and kidney were studied. Only CAT activity, gene expression and protein abundance increased in the kidney	Maisanaba et al. (2014e)
- Clay1-Poly(lactic)acid nanocomposite extract	Hexadecyltrimethyl ammonium bromide (HDTA)	Male Wistar rats	As beverage 90 days	100% extract in water	No evidence of damage in any of the biomarkers studied: histopathology, oxidative stress endpoints (LPO, SOD, CAT, GPx, GST), clinical biochemistry parameters, IL-6 leakage, GSH/GSSG levels	Maisanaba et al. (2014f)

derive from non-intentional sources, such as waste from the manufacturing process, polymer degradation, or landfills (Taylor et al., 2014).

There are different scientific reports evaluating the toxic effects of clay minerals on different animal species, most of which were performed under laboratory conditions. These studies, although still scarce, show contradictory results, with some of them indicating the absence of damage, whereas other investigations report positive effects or the absence of toxicity.

Thus, for example, different bentonite clays have been assayed to establish a toxicological profile. Sodium bentonite was demonstrated to be harmless after 24 h when added at a concentration of 7.5 g/L in 23 fish species (Daugherty, 1951). In contrast, Wyoming bentonite presented a LC50 of 19 g/L after 96 h of exposure in rainbow trout (Sprague and Logan, 1979). Sigler et al. (1984) found, in laboratory studies, that turbidity caused by bentonite clays reduced the growing rate of two different salmon species. In addition, bentonite has demonstrated its effectiveness against the parasitic trematode *Schistosoma mansoni* through adhesion to its surface, reducing its mobility (Olsen, 1987). Moreover, the modified clay Phoslock®, which is bentonite clay in which the sodium and/or calcium ions are exchanged for lanthanum (95% bentonite and 5% lanthanum), was evaluated by Stauber (2000), who reported that leachates of the modified clay did not present toxicity in *Melanotaenia duboulayi* juvenile fish after 96 h of exposure. These clay leachates did not affect the growth of the freshwater alga *Selenastrum capricornutum* after 72 h of exposure. On the contrary, this researcher also described that Phoslock® presented a low toxicity on the cladoceran *Ceriodaphnia dubia* after 48 h but a reduced number of young individuals after seven days with a NOAEL of <3.1 g clay/L. Two different studies performed on *Oncorhynchus mykiss* conducted for 48 and 96 h established that minimal risk is derived from the application of Phoslock® at a standard dose (Martin and Hickey, 2004; Watson-Leung, 2009). Similarly, other studies have demonstrated that even long exposure times or high concentrations of Phoslock® did not produce a significant impact on different benthic invertebrates, such as *Chironomus zealandicus*, *Chironomus dilutes*, *Polypedilum parvidum*, *Hyalella azteca*, *Hexagenia* sp., and *Daphnia magna* (Clearwater, 2004; Clearwater and Hickey, 2004; Lürling and Tolman, 2010; Watson-Leung, 2009). In another study performed by Oosterhout and Lürling (2011), Phoslock® induced toxic effects on a *Daphnia galeata* population, although these researchers stated that these toxic effects could be due to several factors acting in synergy. Other researchers studied the species sensitivity distribution of 12 species and four taxonomic groups exposed to bentonite, concluding that the organisms living in sediments are accommodated to address these clays at high density (Smit et al., 2008). Strachan and Kingston (2012) observed that different species of bivalves (*Modiolus modiolus*, *Venerupis senegalensis*, *Dosinia exoleta*, and *Chlamys varia*) increased their normal filtration rate when bentonite was added to the aqueous media. Moreover, the survival of *D. exoleta* and *V. senegalensis* resulted to be compromised during the exposure time (28 days). Robinson et al. (2010) showed that Mt was more toxic than kaolinite by exposing *D. magna* to different doses of the clay, demonstrating a dose-dependent survival. Concerning plants, Asli and Neumann (2009) demonstrated that the presence of bentonite in water supplies could lead to an accumulation of this clay on the cell wall surfaces of the primary root of *Zea mays* seedlings and consequently induced an inhibition of the cell wall pore size, water transport capacity, leaf growth and transpiration. Recently, EFSA (2013) considered a 0.5% bentonite concentration in animal feeding to be safe for all animal species considered, *i.e.*, chickens, ruminants and trout.

The environmental impact of kaolinite has also been assayed to

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establish safe levels on its uses, although data are still scarce. Thus, Lee (1976) found that the 24- and 48-h LC50 values for kaolinite to *D. pulex* were >1.1 g/L. In addition, different concentrations of kaolinite caused $<10\%$ mortality in some different marine species within 5–12 days (McFarland and Peddicord, 1980). Isono et al. (1998) demonstrated that the hatching success and developmental rates of eggs of four marine fish species (*Pagrus major*, *Acanthopagrus schlegelii*, *Oplegnathus fasciatus*, and *Parapristipoma trilineatum*) was not significantly affected after 24 h of exposure to 10 g/L kaolinite. On the contrary, the latter authors also described larvae mortality in all of the fish species exposed to different concentrations of the kaolinite suspension (0.032–10 g/L) for up to 12 h. Similarly, Robinson et al. (2010) described a decrease in survival after 24 h of exposure of *D. magna* to a double pulse of 734 mg/L kaolinite.

Another clay mineral, sepiolite, has also been evaluated considering its benefits in the animal feeding industry. Thus, it has been evaluated in chickens for fattening, laying hens, pigs and rabbits, highlighting the absence of adverse effects. EFSA concludes that sepiolite, used individually at concentrations of up to 2% complete feed, is safe for all animal species (EFSA, 2013).

Clay minerals have also demonstrated their utility against bacteria, mycotoxins and metal absorption in livestock, such as chickens and piglets (Cabañero et al., 2005; Trckova et al., 2014; Wang et al., 2012b).

Taking these data into account, it is also important to remark that many clay minerals are usually combined with surfactants/modifiers to improve their properties. These compounds can also exhibit toxic effects in animal and plants, as has been stated by some researchers (Garcia et al., 2001; Nalecz-Jawecki et al., 2003; Sarkar et al., 2013; Mori et al., 2015). For this reason, it is important to evaluate the toxicity of clay minerals, the modifiers and their combination.

4.2. Human exposure

The exposure of the general population to low clay concentrations is frequent (López-Galindo et al., 2007); however, it has markedly increased over the last century due to anthropogenic sources (Oberdörster et al., 2005). Clay minerals occur everywhere, including in rocks of all types (Moll, 2000; Pédro, 1994), dispersed in atmospheric aerosols (Sanfeliu et al., 2002), and in water from oceans, seas, lakes, lagoons, and rivers (Gomes, 1987). Several diseases have been attributed to the human intake of polluted air and water (Gomes and Silva, 2007). Inhalation is the most common route of entrance of clay minerals into the organism, but its ingestion and absorption by the intestinal tract and dermal penetration also have to be highlighted (Zhao and Castranova, 2011; Silvestre et al., 2011). Nonetheless, there is currently limited information on occupational exposure to clay minerals in mines, processing plants, and industries. However, it is known that high levels of or inappropriate exposure to clay minerals in the environment and humans may lead to undesirable effects (Zhu and Njuguna, 2014).

Regarding to oral exposure, low doses of clay minerals are used in food preparation (Murray, 2000), in pharmaceuticals for oral administration (Carretero, 2002; Droy-Lefaix and Tateo, 2006), and as herbalist remedies (Mascolo et al., 1999; Tateo et al., 2001). They are used as gastrointestinal protectors (kaolinite and palygorskite), antacids (smectites and palygorskite), and antidiarrhoeaics (kaolinite, smectites and palygorskite). The fundamental properties for which clay minerals are used in pharmaceutical formulations are high specific area and sorptive capacity, favourable rheological characteristics, chemical inertness, low or null toxicity for the patient, and low price (Carretero et al., 2006). Moreover, due to their adsorptive properties, clays such as Novasil have been included in the diet to protect humans against aflatoxins in populations at high risk of aflatoxicosis (Afriyie-Gyawu et al., 2008; Phillips et al., 2008). But, in some cases, clay minerals could be dangerous when ingested (Mascolo et al., 1999; Tateo et al., 2001; Tateo and Summa, 2007; Willhite, et al., 2012). The practice of drinking clay suspensions can be considered a sort of geophagia (soil ingestion), which is a very ancestral and debated human behaviour (Reilly and Henry, 2000; Woywodt and Kiss, 2002; Wilson, 2003; Ziegler, 1997). Geophagia is not only a voluntary ingestion of clay or soil but has a more general meaning because it also includes inadvertent ingestion with possible hazardous implications (Sheppard, 1998; Simon, 1998). Whilst geophagia is recognized as a multi-causal behaviour, a prevalent explanation is the 'nutritional hypothesis' where the deliberate consumption of soil is attributed to an attempt to regulate a mineral nutrient imbalance such as sodium deficiency. Thus, the habit of eating soil can lead to increased exposure and metal toxicity (Mwalongo and Mohammed, 2013). However, paradoxically, some ingested soils can also result in mineral nutrient deficiency problems attributable, for example, to the adsorptive properties of earth materials that can effectively bind chemical elements (Abrahams 2012).

Ingested soils can have a significant impact on the mineral nutrition of humans being potentially a source and/or sink of essential elements and potentially harmful elements (Hooda et al., 2004; Abrahams 2012).

The pathogenicity of clay minerals is mainly caused by inhalation (Carretero et al., 2006). Inside the lung, clay minerals can cause diverse pathologies, such as cancer, mesothelioma, and pneumoconiosis, but the toxicity of these minerals is generally related to both the presence of quartz or asbestos from mining works and with the geological conditions of formation (Carretero et al., 2013). However, calcium Mt in the absence of quartz has been reported to induce pneumoconiosis, characterised microscopically by grey black stellate nodules and microscopically by centrilobular intersticial collections of brown dust laden macrophages associated with slight fibrosis (Gibbs and Pooley, 1994).

Bentonite is a widely distributed material that occurs abundantly as dust and near surface deposits of bentonite and is widely dispersed by air and moving water (Parker, 1988). The expected acute oral toxicity of bentonite in humans is very low ($LD50 > 15$ g/kg) (HSDB, 2014). However, long-term occupational exposures to bentonite can cause pneumoconiosis, which is a chronic inflammatory and fibrotic lung disease, in workers involved in mining and processing (Cui and Zhang, 2001; Gibbs and Pooley, 1994; Phibbs et al., 1971). Kondej and Sosnowski (2013) reported that aluminosilicate nanoparticles (bentonite, halloysite, and Mt) in the pulmonary liquid above 0.1 mg/cm^3 were capable of promoting alterations of the original dynamic biophysical activity of a model pulmonary surfactant and that this effect was indicated by deviation of the minimum surface tension, stability index and the size of surface tension hysteresis.

In general, however, there is a concomitant exposure to other minerals (*e.g.*, silica and amphiboles), and the response to these minerals complicates the interpretation of the data. For example, bentonite deposits generally contain other minerals, including very fine-grained quartz and amorphous silica. In the case of Wyoming bentonites, the silica content (which includes both quartz and cristobalite) ranges from 0% to 24% (Ross et al., 1993). Sampatakakis et al. (2013) performed the first epidemiological study in which a specific methodology is used to indicate the dust role of bentonite and perlite in the mortality and morbidity rates of permanent residents (Milos Island). In these residents, an increased risk of developing diseases of the respiratory system, such as pneumonia, chronic obstructive pulmonary disease and allergic rhinitis, has been observed. The morbidity of these diseases was significantly elevated. Moreover, Yuwen et al. (2013) investigated

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the genetic damage and LPO in workers exposed to bentonite by inhalation. The results showed that excessive occupational exposure to this clay mineral can lead to detectable genetic damage and LPO, which may be influenced by the air exposure concentrations of organic bentonite particles. However, bentonite is not considered toxic to humans; thus, this clay mineral has been approved as a food additive in different countries, such as Australia (NICNAS, 2001) and Europe (EFSA, 2013). In this sense, Afsar and Groves (2009) observed that Phoslock® application to a water body to remove phosphorus and eliminate the presence of blue-green algal blooms did not cause damage to human health.

Kaolin and the clay mineral kaolinite are natural components of soil and occur widely in ambient air as floating dust. Accordingly, the exposure of the general population to these materials must be universal, albeit at low concentrations. In the vicinity of mines and industrial projects, kaolinite is likely to be present at high concentrations in air. The composition of the clay, *i.e.*, the quantity and quality of minerals other than kaolinite, is an important determinant of the effects (Adamis et al., 2005). The clay mineral kaolinite has been detected in the lung tissue of bronchoalveolar lavage fluid of various worker groups (Churg, 1983; Churg and Wiggs 1985; Dumortier et al., 1989; Johnson et al., 1986). Mastin et al. (1986) also evidenced the presence of inorganic particles in the lungs of non-occupationally exposed population. Quartz, talc, and kaolinite could be detected in the lungs of practically all subjects. Paoletti et al. (1987) identified 17 different mineral types in the lung parenchyma of 10 deceased subjects living in an urban area with no occupational dust exposure. Approximately 70% of the minerals consisted of phyllosilicates, particularly micas, clays (kaolin and pyrophyllite) and chlorites. In addition, several different mineral types have been detected in the lungs of 11 unselected autopsy cases, including quartz, feldspar, kaolinite, and mica (Kalliomäki et al., 1989). Kaolinite particles constituted $10 \pm 5\%$ of the mineral particles in the lungs. More mineral particles were also found in the lungs of non-smokers, but the differences were not statistically significant. Similarly, Yamada et al. (1997) also detected (fibrous) talc, mica, silica, chlorite, and kaolinite particles in the lungs of female lung cancer patients and residents from urban and rural areas in Japan.

Epidemiological studies, as reviewed by Guthrie (1992), suggest that kaolinite-bearing dust is fibrogenic only under extraordinary conditions, notably high concentrations of dust or exposure combined with another respiratory disease, such as tuberculosis. Other researchers have indicated that excessive exposure to kaolinite dust may lead to the development of pneumoconiosis (Davis and Cotton, 1983; Kennedy et al., 1983; Levin et al., 1996; Ross et al., 1993; Sepulveda et al., 1983; Wagner et al., 1986). However, pulmonary fibrosis has been found in kaolinite workers in America, where the mica content is negligible (Lapenas and Gale, 1983; 1984).

Another clay is illite, which is widely distributed in nature, abundant, and often the dominant mineral clay in soil, terrestrial deposits, sedimentary rocks, freshwater sediments, and most deep-sea clays (Grim, 1968). Only a few epidemiological studies of respiratory disease resulting from exposure to dusts containing illite and smectites have been published to date. Some researchers have suggested that inhaled smectite is likely to be less dangerous to humans than kaolin (López-Galindo et al., 2007).

In contrast, chronic exposures to high concentrations of talc were associated with the development of talcosis, a type of pneumoconiosis (Carretero et al., 2006). Although it occurs commonly as an occupational hazard, pulmonary disease due to talc is more often encountered after the intravenous administration of talc during drug abuse (Neuman et al., 2011). Gibbs et al. (1992) observed cases of "talc pneumoconiosis". Mixed dust fibrotic lesions were found in two cases in which there were substantial quantities of quartz present. Great variations in the minerals were found within the lung tissues. This study showed that "talcosis" frequently represents a disease associated with a variety of minerals and that talc is a common denominator. Neuman et al. (2011) presented a case report of a worker who was exposed to talcum during his work in tire manufacturing. During his lifetime, an occupational disease was not recognised. The autopsy revealed lung fibrosis. Based on the pathological and mineralogical findings, the confirmed occupational exposure toward talc and the exclusion of other possible causes (asbestos and quartz), the diagnosis of a talc-induced interstitial lung fibrosis, namely talcosis, was established. Nath et al. (2014) presented a case of talcosis in a worker who had considerable exposure to talc during the initial preparation of "samosa," a triangular fried Indian savoury. Typical pulmonary histological findings of talcosis with extensive deposition of birefringent crystals in histiocytes were observed. Talcosis in the course of food adulteration is seldom or hardly reported.

5. Conclusions

The improvements that clay minerals used in technical areas provide are well known, but their toxicity profile currently has many gaps. In this sense, the variety of the toxicity results described in the scientific literature is huge, as was shown throughout the review. Thus, most of *in vitro* toxicity reports showed cell death and different toxic mechanisms involved (genotoxicity, oxidative stress, *etc.*) whereas human and animal data showed a lower toxicity. Therefore, it is difficult to reach a definitive conclusion regarding the toxicity or security of clays and their uses. Additionally, few exhaustive comparative studies have been performed. It is important to note that the toxicity profiles of clay minerals and derived nanocomposites are defined according to different parameters, including (i) the exposure conditions, such as the concentrations or exposure times assayed, (ii) the experimental models selected, (iii) the modifiers or surfactants incorporated in their structures and their concentrations and (iv) the sensitivity of the assays performed.

In conclusion, many benefits could be derived from clay minerals and their products, but a case by case toxicological evaluation is always required to avoid potential human and environmental risks.

Acknowledgements

The authors thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Consejería Economía, Innovación, Ciencia y Empleo, Junta de Andalucia (AGR5969) for the financial support for this study. Sara Maisanaba also gratefully acknowledges Junta de Andalucia for her predoctoral grant associated to the AGR5969 project.

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Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

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Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

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Please cite this article as: Maisanaba, S., et al., Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environ. Res. (2015), http://dx.doi.org/10.1016/j.envres.2014.12.024

CAPÍTULO 2 / CHAPTER 2

Sara Maisanaba, María Puerto, Silvia Pichardo, María Jordá, F. Javier Moreno, Susana Aucejo, Ángeles Jos

IN VITRO TOXICOLOGICAL ASSESSMENT OF CLAYS FOR THEIR USE IN FOOD PACKAGING APPLICATIONS

Food and Chemical Toxicology 57, 266-275, **2013**

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Highlights

• Clays used in food applications induce cytotoxic effects in HepG2 cell line. • The modifiers of the clays have a role in their toxicity. • Cloisite 30B induces cyto and genotoxicity, GSH decrease and morphological damage.

Food and Chemical Toxicology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

In vitro toxicological assessment of clays for their use in food packaging applications

Sara Maisanaba[a], María Puerto[a], Silvia Pichardo[a], María Jordá[b], F. Javier Moreno[c], Susana Aucejo[b], Ángeles Jos[a,*]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville, Spain
[b] Packaging, Transport, & Logistics Research Institute (ITENE), C/Albert Einstein 1, Parque Tecnológico, Paterna, Spain
[c] Area of Cellular Biology, Faculty of Biology, University of Seville, Avda. Reina Mercedes s/n, 41012 Seville, Spain

ARTICLE INFO

Article history:
Received 21 January 2013
Accepted 29 March 2013
Available online xxxx

Keywords:
Clay
Montmorillonite
Cytotoxicity
HepG2 cell line

ABSTRACT

Montmorillonite based clays have a wide range of applications that are going to contribute to increase human exposure to these materials. One of the most promising uses of clays is the development of reinforced food contact materials that results in nanocomposites with improved barrier properties. Different organoclays have been developed introducing modifiers in the natural clay which is commercially available. However, the toxicological aspects of these materials have been scarcely studied so far. In the present study, the cytotoxic effects of a non-modified clay (Cloisite® Na+) and an organoclay (Cloisite® 30B) have been investigated in the hepatic cell line HepG2. Only Cloisite® 30B showed cytotoxicity. In order to elucidate the toxic mechanisms underlying these effects, apoptosis, inflammation, oxidative stress and genotoxicity biomarkers were assayed. Moreover, a morphology study with light and electron microscopy was performed. Results showed genotoxic effects and glutathione decrease. The most relevant ultraestructural alterations observed were mitochondrial degeneration, dilated endomembrane systems, heterophagosomes formation, fat droplets appearance and presence of nuclear lipid inclusions. Cloisite® 30B, therefore, induces toxic effects in HepG2 cells. Further research is needed to assess the risk of this clay on the human health.

1. Introduction

Clays have an array of commercial applications in different fields: the manufacture of inks, paints, greases and cosmetics, water treatment processes, the controlled release of therapeutic agents, food packaging, etc. (Lordan et al., 2011). In regard to food packaging applications, clays are used to improve the barrier properties of food contact materials. This results in a length of the storage time while keeping the product fresh. Polymers incorporating clay nanoparticles are among the first polymer nanocomposites to emerge on the market as improved materials for food packaging. This is due to the easy availability of the raw clay materials and because their cation exchange chemistry has been intensively studied. In addition, these clay nanoparticles have unique properties such as large surface areas, large aspect ratios and improved mechanical, thermal and optical properties (Utracki and Kamal, 2002; Pavlidou and Papaspyrides, 2008). The most frequently used clay in the preparation of polymer nanocomposites is montmorillonite, which is the major constituent of bentonite. Montmorillonite is a natural clay which occurs as plate-like particles called platelets. These platelets have an average thickness of only 1 nm, while its dimensions in length and width can be measured up to 1 mm (Lordan et al., 2011). One limitation of clays is the incompatibility between the hydrophilic clay and a hydrophobic polymer, which could cause agglomeration of clay in polymeric matrices (Elmore and Andersen, 2003; Zeng et al., 2005). Therefore, surface modification of clay minerals is an important step to achieve polymer nanocomposites. By cation exchange with organic cations, clays become hydrophobic and thereby compatible with polymers. Such modified clays are referred to as organoclays (Sharma et al., 2010). The most widely known theories to explain the improved barrier properties of polymer–clay nanocomposites are based on a theory developed by Nielsen (1967), which focuses on a tortuous path around the clay plates, forcing the gas permeant to travel a longer path to diffuse through the film.

Successful technical development of nanocomposites for food packaging has to overcome barriers in safety, technology, regulation, standardization, etc. (Silvestre et al., 2011). Regarding to safety, since the use of clays and organoclays is increasing, it is important to take into account their toxicity. Clays are natural materials but they are not free of possible side-effects. In this sense, toxicity studies of clays have been scarcely performed. The toxicological evaluation of these products can be faced using both

* Corresponding author. Tel.: +34 954 556762; fax: +34 954 556422.
E-mail address: angelesjos@us.es (Á. Jos).

0278-6915/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fct.2013.03.043

in vivo and *in vitro* methods. The primary aim of *in vitro* testing is toxicity screening and the understanding of biological responses and underlying mechanisms (EFSA, 2011). Moreover, *in vitro* methods are encouraged in toxicological research for ethical reasons.

Toxic effects of clay minerals have been shown to occur mainly after inhalation (Carretero et al., 2006; Sharma et al., 2010). But also, one of the most likely routes of exposure to these clays for the general population is the oral pathway, since they are present in food contact materials. Tateo and Summa (2007) reported that the ingestion of clays is common at low doses in food preparations, in pharmaceuticals for oral administration, and as herbal remedies. Moreover, distribution studies performed with different nanoparticles showed that they can translocate to several organs such as liver, kidney, lungs. (Kim et al., 2008; Kwon et al., 2008; EFSA, 2009) so for clays this possibility cannot be discarded.

In view of the limited toxicological information of clays, in this study we aim to evaluate the toxicity of an unmodified (Cloisite® Na+) and an organomodified clay (Cloisite® 30B) in the human hapatocellular cell line HepG2. For this purpose, basal cytotoxicity biomarkers and mechanistic biomarkers of oxidative stress, inflammation and genotoxicity have been investigated. Moreover, a histopathological study has been also performed.

2. Materials and methods

2.1. Clay materials and characterization

Unmodified montmorillonite (Cloisite® Na+) and the organically modified one (Cloisite® 30B) were obtained from Southern Clay Products, INC. (modifier: methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium, concentration: 90 meq/100 g clay). Both clays were characterized by thermogravimetric analysis (TGA), X-ray diffraction (XRD) and particle size distribution (PSD).

TGA analysis of Cloisite Na+ and Cloisite 30B were performed on a Q5000IR thermobalance (TA Instruments) by heating the samples from room temperature up to 900 °C with heating rate of 10 °C/min, in nitrogen atmosphere. Approximately 7 mg of each finely ground sample was heated in a platinum crucible. Powder XRD analyses were performed using a D8ADVANCE A25 Bruker diffractometer. The clay powders were mounted on a sample holder with a large cavity and a smooth surface was obtained by pressing the powders with a glass plate. The X-ray diffraction patterns were measured from 1.5° to 25° (2θ) at a scan rate of 0.02 s. Particle size distributions of the samples were estimated with a Mastersizer 3000 (Malvern Instruments Ltd., UK). Dispersion in dry basis was the selected method to perform the different assays, with controllable airflow and feed rate. This method allows measuring particle sizes between the range 0.1–3500 μm.

2.2. Clays test solutions

The test concentrations for both clays were selected taking into account previous dispersion experiments in order to avoid interferences with the measurement system. The highest concentrations tested were 62.5 and 500 μg/mL for Cloisite® Na+ and Cloisite® 30B, respectively. Test solutions were prepared in serum-free medium. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

2.3. Cell culture

HepG2 (human hepatocellular carcinoma epithelial cell line) (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% of fetal calf serum (FCS, Gibco, New Zealand), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, New Zealand). Cells were grown at 37 °C and 5% CO_2 in a humidified atmosphere.

2.4. Cytotoxicity assays

For cytotoxicity assays, exposure concentrations for Cloisite® Na+ were set at 0, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5 μg/mL and for Cloisite® 30B at 0, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 μg/mL. HepG2 cells were seeded at a density of 7.5×10^5 cell/mL in 96 wells plates and exposed to the clays for 24 and 48 h.

Total cellular protein content (PC) was quantified following the method of Bradford (1976). After exposure, cells were washed with phosphate buffer saline (PBS) and incubated with 0.1 N NaOH for 2 h at 37 °C. Later, a 22% Coomassie brilliant blue solution (Biorad, Spain) was added to the wells and within 30 min absorbance was read at 595 nm on a microplate reader (Biotek, USA).

Neutral Red (NR) uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, NR in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. NR can be extracted from lysosomes for quantitative measurement at 540 nm.

The MTS tetrazolium reduction assay was performed according to a procedure based on Baltrop et al. (1991), being MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt bioreduced by cells into a colored formazan product soluble in culture medium. Cells were directly incubated with MTS tetrazolium salt for 2 h at 37 °C and absorbance was read at 492 nm.

Only when cytotoxicity was observed, mechanistic biomarkers were determined. In this case, the mean effective concentration (EC_{50}) of the most sensitive cytotoxicity endpoint was chosen as the higher exposure concentration to investigate mechanistic biomarkers along with the fractions $EC_{50}/2$ and $EC_{50}/4$.

2.5. Caspase-3/7 activity

Caspase-3 and -7 activities were used as apoptosis biomarkers in cells exposed for 24 and 48 h to the clays. Manufacturer instructions from the kit (Caspase-Glo® 3/7 Assay, Promega, USA) were followed.

2.6. ROS generation

The production of ROS was assessed in 96 wells microplates using the dichlorofluorescein (DCF) assay. Cells were incubated with 200 μL, 40 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in culture medium at 37 °C for 30 min, and then washed with PBS and resuspended in 200 μL of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as fluorescence arbitrary units (Puerto et al., 2010).

2.7. GSH content

Cellular glutathione (GSH) content was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl) (Jos et al., 2009). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme glutathione-S-transferase (GST). After the cell exposure the medium was discarded and cells were incubated at 37 °C for 20 min in the presence of 40 μM mBCl. Later on cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the following excitation/emission wavelengths: 355/460. Results were expressed as fluorescence arbitrary units.

2.8. Interleukin-6 leakage

For this assay the culture medium of the cells after 24 and 48 h exposure to the clays was used. Manufacturer instructions from the kit (EH2IL6, Thermo Scientific, USA) were followed.

2.9. Comet assay

The comet assay was performed to detect DNA strand breaks. HepG2 cells were seeded into 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37 °C in 5% CO_2 to attach to the plates. Approximately 3.5×10^5 cells in each well were exposed with different concentrations of Cloisite® 30B (0, 22, 44 or 88 mg/mL) after 24 and 48 h to exposure.

In order to monitor the ongoing process of the assay, a negative control (cells treated with medium without fetal calf serum) and a positive control (cells treated with a solution of 100 μM H_2O_2) were included. After treatments cells were washed and detached in PBS. The comet assay was applied as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, cells were resuspended in PBS at a concentration of 2.5×106 cells/mL. This suspension were mixed with 1% low melting point agarose and placed on a microscope slide. Once the gels had become solid, the slides were dipped into lysis solution at 4 °C. All nucleotides were denatured in a high-pH buffer. Electrophoresis was carried out approximately at 25 V (300 mA) and the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold nuclei acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20× objective) coupled via a CCD camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei (⩾100) per experimental point were analyzed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK).

Please cite this article in press as: Maisanaba, S., et al. *In vitro* toxicological assessment of clays for their use in food packaging applications. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.03.043

2.10. Morphology

Cells were exposed to different concentrations of Cloisite® 30B (0, 22, 44 or 88 mg/mL) during 24 and 48 h of exposure. Afterwards, cultured cells were fixed directly in the cell culture dish in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4 °C. They were all postfixed in 1% osmium tetroxide for 60 min at 4 °C. Samples were dehydrated in ethanol at progressively higher concentrations and embedded in Epon (epoxy embedding medium). Toluidine blue-stained semithin sections (0.5 μm thick) used as controls were viewed in a Leitz (Aristoplan) light microscope. Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope.

2.11. Calculations and statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $p < 0.05$. EC_{50} values, mean effective concentration, concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls, were determined by linear interpolation.

3. Results

3.1. Characterization of Cloisite® Na+ and Cloisite® 30B

TGA results of both clays are presented in Fig. 1. Loss weight percent (%), and derivative weight (%/°C) are presented versus temperature. It can be observed that only the sample Cloisite® 30B has big loss step in the range between 200 and 500 °C, indicating that an organic compound is being released in this step. The percentage of organic modifier in Cloisite® C30B is 35.98%, as calculated in the TGA curves. Cloisite® Na+ hardly presents any weight change in this range of temperature.

XRD results of both clays are presented in Fig. 2. The signal intensity is plotted versus de 2θ. Results show the typical patterns associated with a montmorillonite material. The main difference corresponds to the signal associated to the interlayer space in the clays (corresponding to the d(001) diffraction peak); in the case of Cloisite® Na+ is 8.8 (2θ), and in the case of Cloisite® 30B is 4.7 (2θ). The distances of silicate layers can be calculated by Bragg's Law ($n\lambda = 2d\sin\theta$, d = layer distance). The measured $d001$-spacing of Cloisite® Na+ is 10.0 Å ($2\theta = 8.8$), but after cation exchange with the modifier (Cloisite® 30B) $d001$-spacing became 18.7 Å ($2\theta = 4.7$).

PSD of the samples has been calculated and results are presented in Table 1. It can be observed that particle size of Cloisite® 30B is lower for all the values (Dv10, Dv50, and DV90) than those of Cloisite® Na+.

3.2. Cytotoxicity assays

Cloisite® Na+ did not induce cytotoxicity after the exposure of the cells for 24 and 48 h at the concentrations assayed (Fig. 3). Only the PC assay showed a small significant reduction at the higher concentration (62.5 μg/mL). The EC_{50} could not be calculated in any of the biomarkers.

Protein content was the less sensitive endpoint of Cloisite® 30B cytoxicity. After 24 h exposure to Cloisite® 30B, this endpoint decreased from 250 μg/mL, with a calculated EC_{50} of 285 ± 9 μg/mL. Similar reductions of protein content were remarkable after 48 h, although after 2 days of exposure, the decrease could be appreciable already from 125 μg/mL in comparison to 24 h (Fig. 4a). Regarding to NR uptake, no significant changes were observed in the four lower concentrations used, although a concentration-dependent decrease was observed from 62.5 μg/mL with EC_{50} values of 88 ± 4 and 55 ± 3 μg/mL at 24 and 48 h, respectively (Fig. 4b). MTS metabolization decreased substantially, with a reduction of around 90% at the higher concentration tested compared to the control. After 48 h, MTS metabolization was significantly reduced between 62.5 and 500 μg/mL. EC_{50} values for this endpoint varied between 158 ± 7 and 79 ± 5 μg/mL after 24 h and 48 h respectively (Fig. 4c).

The most sensitive endpoint for Cloisite® 30B was the neutral red uptake, therefore the EC_{50} value of this biomarker (88 μg/mL) was chosen as the higher exposure concentration for the mechanistic studies in the human hepatocellular cell line along with the fractions $EC_{50}/2$ and $EC_{50}/4$, being the concentrations used 88, 44 and 22 μg/mL.

3.3. Caspase-3/7 activity

The caspase activity assay did not show any significant variation with any of the concentrations of Cloisite® 30B and at any time of exposure (Fig. 5).

3.4. ROS and GSH content

When HepG2 cells were exposed to Cloisite® 30B during 24 and 48 h, no significant alteration on ROS was observed at any of the

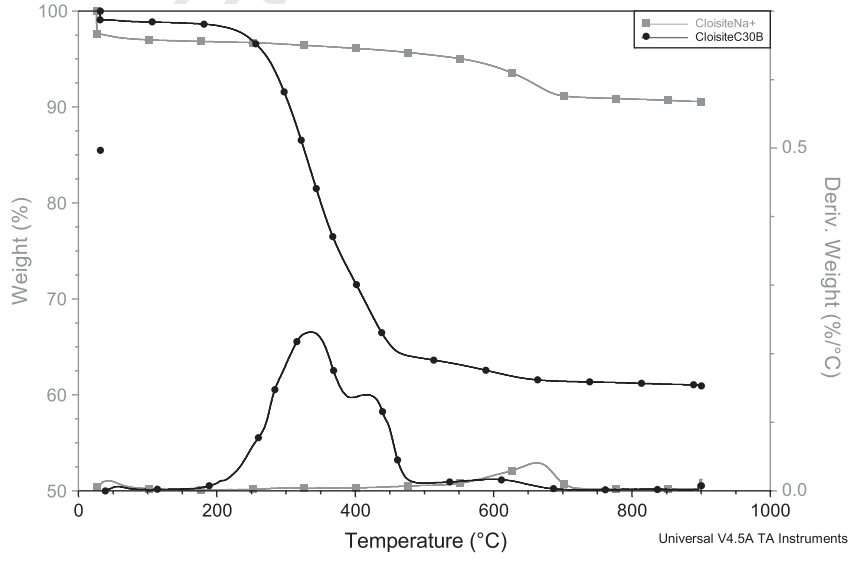

Fig. 1. TGA results for Cloisite® Na+ (square) and Cloisite® 30B (round).

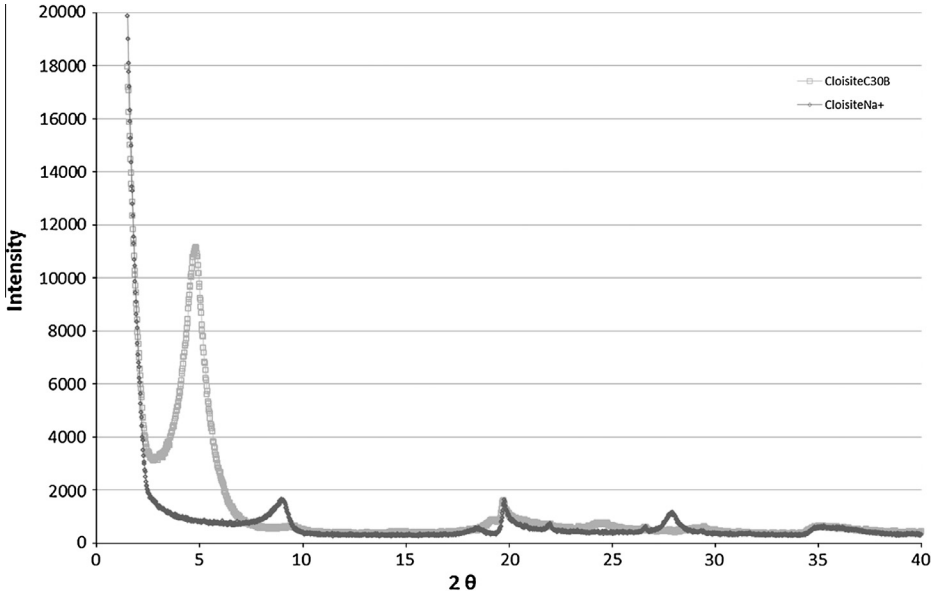

Fig. 2. DRX results for Cloisite® Na+ (rhombus) and Cloisite® 30B (square).

Table 1
PSD results for Cloisite® Na+ and Cloisite® 30B.

Sample	Dv 10 (μm)	Dv 50 (μm)	Dv 90 (μm)
Cloisite® Na+	5.33	15.71	33.23
Cloisite® 30B	2.63	9.20	24.58

exposure concentrations in comparison to the control group (Fig. 6a). In contrast, a concentration-dependent decrease of GSH was observed being the GSH content 10-fold lower at the higher concentration assayed. Moreover, there were not statistical differences with respect to the control in any concentration tested and times of exposure (Fig. 6b).

3.5. IL-6 leakage

The IL-6 assay showed no increase in the IL-6 content in the cell culture medium of the cytotoxicity assays in any of the treatments performed (data not shown).

3.6. Genotoxicity: comet assay

Cloisite® 30B induced time-dependent increases of DNA strand breaks in HepG2 cells (Fig. 7). After 24 h of exposure no changes were observed after exposure to the clay; in contrast a significant increase of DNA damage was observed after 48 h incubation with the highest concentration (88 μg/mL). The mean value of the% tail DNA was 23.4, while in the control group was only 2.9%. The percentage of DNA in the tail for 88 μg/mL is not significantly different from the percentage of DNA found for the positive control.

3.7. Morphologic study

3.7.1. Light microscope observations

Control HepG2 cells observed under light microscope are shown in Fig. 8a. After 24 h of exposure to 22 μg/mL Cloisite® 30B, they are still growing with similar morphological characteristics to the control group. However, after 48 h of exposure to 88 μg/mL Cloisite® 30B, a decrease in the growing rate is observed, which could be due to a cellular cycle stop and cell death (Fig. 8b). Moreover, cells show severe morphological changes such as intense vacuolization.

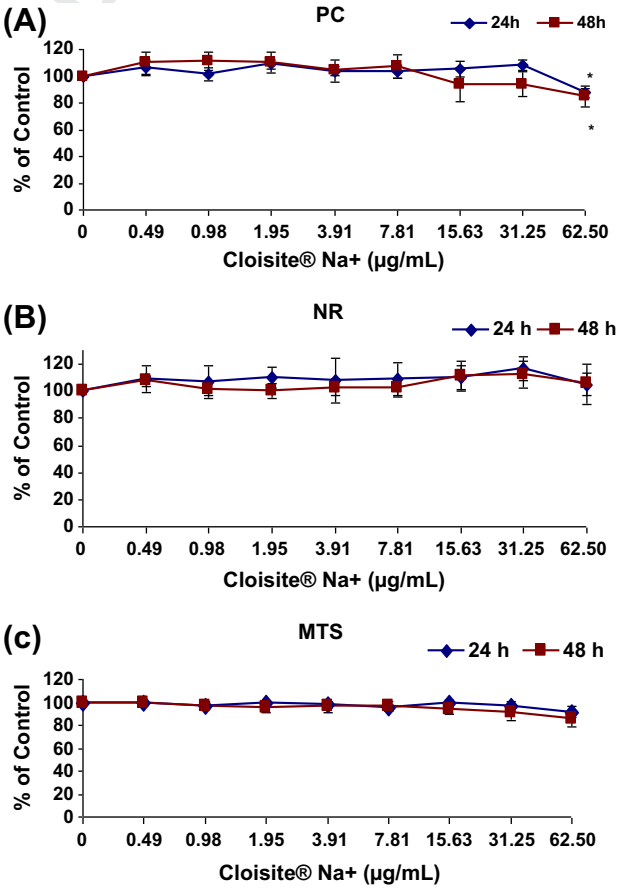

Fig. 3. Protein content, PC (a); neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HepG2 cells after 24 h and 48 h of exposure to 0–62.5 μg/mL Cloisite® Na+. All values are expressed as mean ± SD. *Significantly different from control ($p \leqslant 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

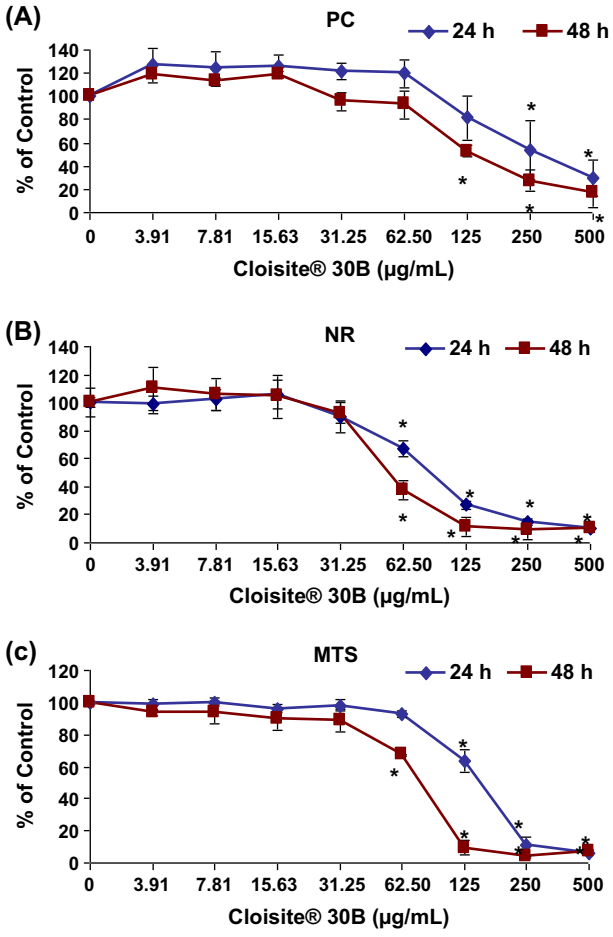

Fig. 4. Protein content, PC (a); neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HepG2 cells after 24 h and 48 h of exposure to 0–500 µg/mL Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($p \leqslant 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

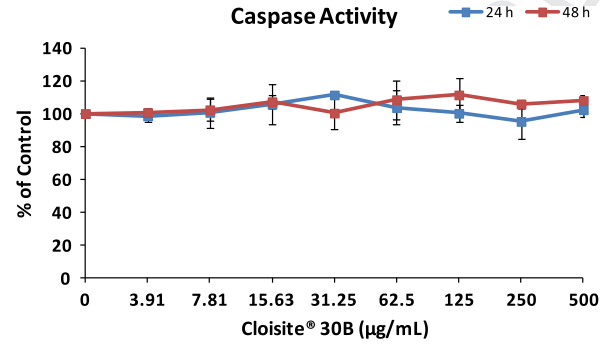

Fig. 5. Caspase-3/7 activity of HepG2 cells after 24 h and 48 h of exposure to 0–500 µg/mL Cloisite® 30B. All values are expressed as mean ± SD.

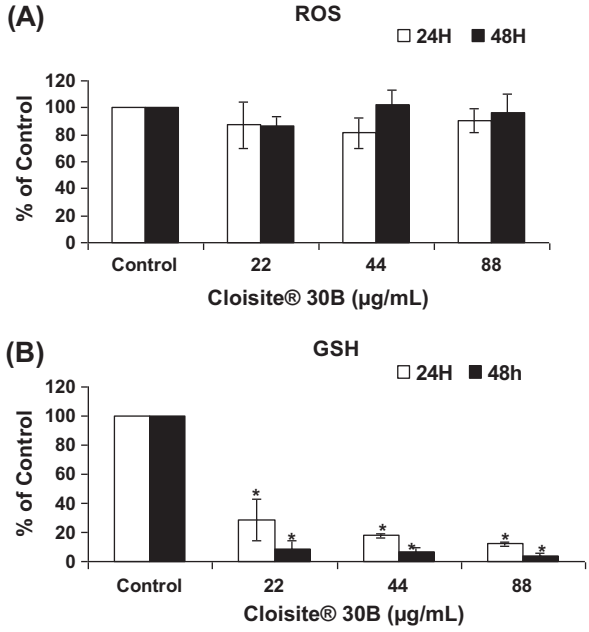

Fig. 6. ROS content (a), and GSH content (b) in HepG2 cells after 24 and 48 h of exposure to 22, 44, or 88 µg/mL Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($p \leqslant 0.05$).

3.7.2. Electron microscope observations

The most remarkable ultrastructural features of unexposed HepG2 cells, observed by electron microscopy, are big irregular nuclei with numerous heterochromatic accumulations dispersed in the nucleoplasm and prominent nucleoli. Moreover, cytoplasm is rich in mitochondria with light matrix, endoplasmic reticulum cisternae and lipid drops (Fig. 9a). In the culture, undifferentiated cells coexist with cells under certain degree of differentiation, which exhibit membrane junction complex characteristic of epithelial cells such as tight junction, adherent junction and desmosomes (Fig 9b). All these junctions allow the development of intracellular spaces which are bounded by cytoplasm projections, similar to microvilli, which could resemble the hepatic bile canaliculi (Fig 9b). After 24 h of exposure to the lowest concentration assayed, 22 µg/mL Cloisite® 30B, cells show heterophagosomes in the cytoplasm and accumulation of secretory material, such as lipid drops, in certain regions of the cytoplasm, displaying cellular polarization (Fig. 9c). Euchromatic nuclei with visible nucleoli are observed, which indicates that cells still maintain their transcriptional activity (Fig. 9c). When cells were exposed to 44 µg/mL Cloisite® 30B for 24 h, nuclei exhibited a higher content of heterochromatin (Fig. 9d). Cells show lipid drops with tendency to confluence, heterophagosomes and mitochondria with dense matrix and visible crest. The most remarkable changes were observed when cells were exposed to the highest concentration, 88 µg/mL Cloisite® 30B (Fig. 9e–g). Cells show dilated endomembrane systems (endoplasmic reticulum cisternae and dictyosomes saccules) (Fig. 9e). Moreover, dense mitochondria underwent morphological changes resulting in dumbbell figures, which are frequently rounded by rough endoplasmic reticulum cisternae (Fig. 9e). In the cytoplasm, confluent lipid drops are abundant as well as heterophagosomes with membranous debris, probably due to autophagic processes. Trans saccules from dictyosomes produce covered vesicles containing hydrolytic enzymes which fuse with the endosomes to form phagosomes (Ph) (Fig. 9e–g).

When HepG2 cells were exposed to 22 µg/mL Cloisite® 30B during 48 h, a greater injure was observed in comparison to the exposure to 24 h (Fig. 10). Most of the cells showed nuclei with irregular borders, heterochromatin accumulations linked to the inner nuclear membrane and nucleoli well developed (Fig. 10a). Rough endoplasmic reticulum cisternae, frequently associated with mitochondria, and dilated dictyosomes are observed in the cytoplasm (Fig. 10a). Mitochondria show a higher size compared to those observed in the control cells, indicating mitochondrial hypertrophy with disorganized crests (Fig. 10a). After 48 h of exposure to 44 µg/mL Cloisite® 30B, cells exhibit a higher chromatin condensation in the nucleus (Fig. 10b). Moreover, cytoplasm is intensively

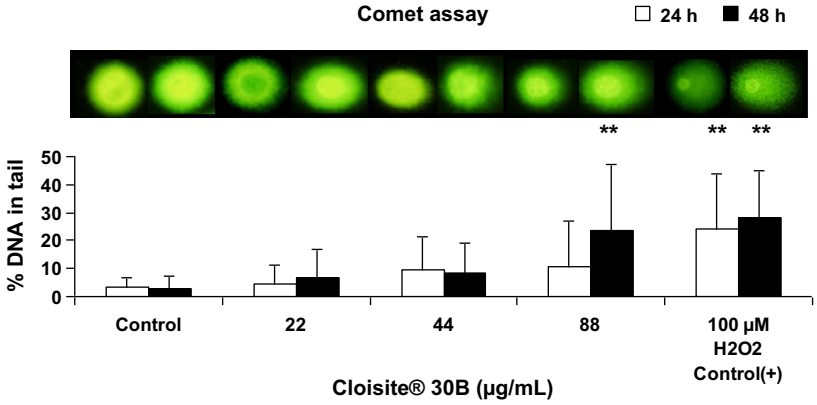

Fig. 7. Comet assay results of HepG2 cells after 24 and 48 h of exposure to 22, 44, or 88 µg/mL Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($p \leqslant 0.05$).

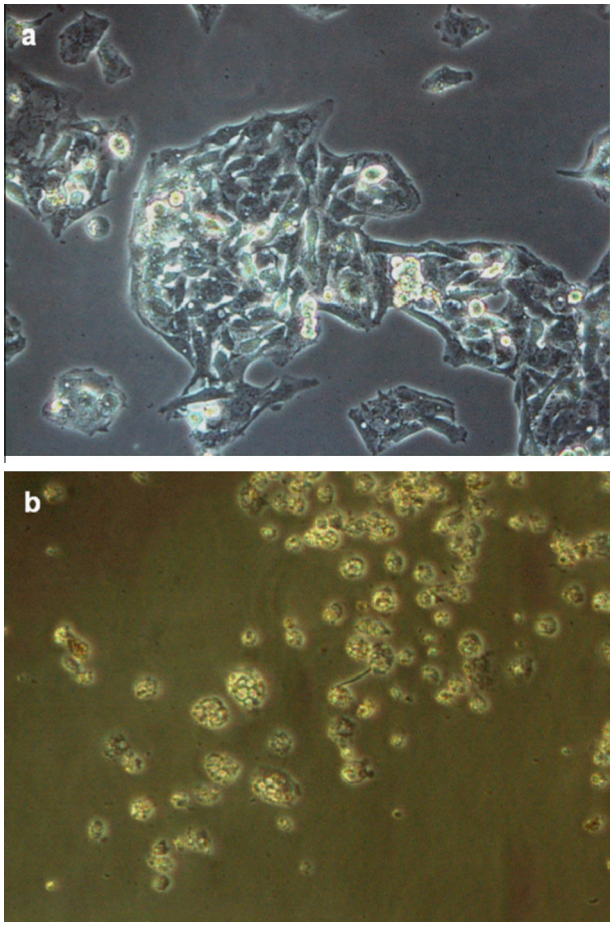

Fig. 8. Morphology of HepG2 cells observed by optical microscopy. Bars = 100 µm. Unexposed control culture (a), and exposed to 88 µg/mL Cloisite® 30B for 48 h (b).

vacuolated with membranous debris inside vacuoles (Fig. 10b). Dilated rough endoplasmic reticulum cisternae associated with hypertrophic mitochondria are still visible (Fig. 10b and c). However, mitochondria morphology changes within time, turning into an elongated and semicircular appearance (Fig. 10c). Phagosomes with membranose debris are also found (Fig. 10c). Near phagosomes, Golgi apparatus with dilated dictyosomes are observed (Fig. 10d). In the trans regions of dictyosomes, vesicles rounded by swollen electrodense membrane can be distinguished, which could probably correspond to vesicles covered by clatrine with high content in hydrolytic enzymes (Fig. 10d). After 48 h of exposure to 88 µg/mL Cloisite® 30B, decrease in the growing rate is observed, which was previously described in the light microscope observation. The surviving cells show highly vacuolated cytoplasm, being these vacuoles big heterophagosomes containing membranous debris, dense bodies and lipid material (Fig. 10e). All these findings are characteristic of autophagic processes and cell death. Moreover, dilated endoplasmic reticulum with big spaces between inner and outer nuclear membrane, mitochondrial degradation, chromatin condensation, and presence of nuclear inclusions, frequently of lipid nature, are observed, which are representative of cell death (Fig. 10f).

4. Discussion

Although the use of clays for developing improved polymer nanocomposites is increasing, there is still very few information in relation to the toxicity these materials can induce. Lordan et al. (2011) also investigated the cytotoxicity of Cloisite® Na+ in HepG2 cells and found a reduction of viability from 1 µg/mL with the MTT assay, a similar endpoint to the MTS assay used in this study. Our results, however, did not show any significant reduction up to 31.25 µg/mL and the highest concentration used was 62.5 µg/mL, because higher ones were observed to interfere with the measurement system. These differences could be derived from different reasons such as the cell density employed for the assays, the origin of the cell culture, etc. Sharma et al., 2010 and Gutiérrez-Praena et al. (2011a), Gutiérrez-Praena et al. (2013), studied the cytotoxicity of the same clay in Caco-2 cells (a human colon-cancer cell line) and no cytotoxic effects were observed by both authors. Moreover, Lordan and Higginbotham (2012) also investigated the cytotoxic effects of Cloisite® Na+ in U937 monocityc cells and the results showed no significant differences with respect to the control. In the case of Cloisite® 30B, a concentration of 500 µg/mL could be assayed and resulted in a total reduction of the viability in any of the biomarkers employed. However, at the concentrations tested this clay did not cause an increase in caspase-3/7 activation. Lordan et al. (2011) obtained similar effects with Cloisite® Na+ and Cloisite 93A and suggested that necrosis was the toxic mechanism involved in the cell death observed. In this case further studies are necessary to elucidate the mechanism of cell death. Cloisite® 30B Q2 has been assayed by Gutiérrez-Praena (2011b; 2013) in Caco-2 cells obtaining also cytotoxic responses. Similarly, Sharma et al. (2010) observed significant differences respect to control at the highest concentrations tested (170 and 226 µg/mL) in this cell line.

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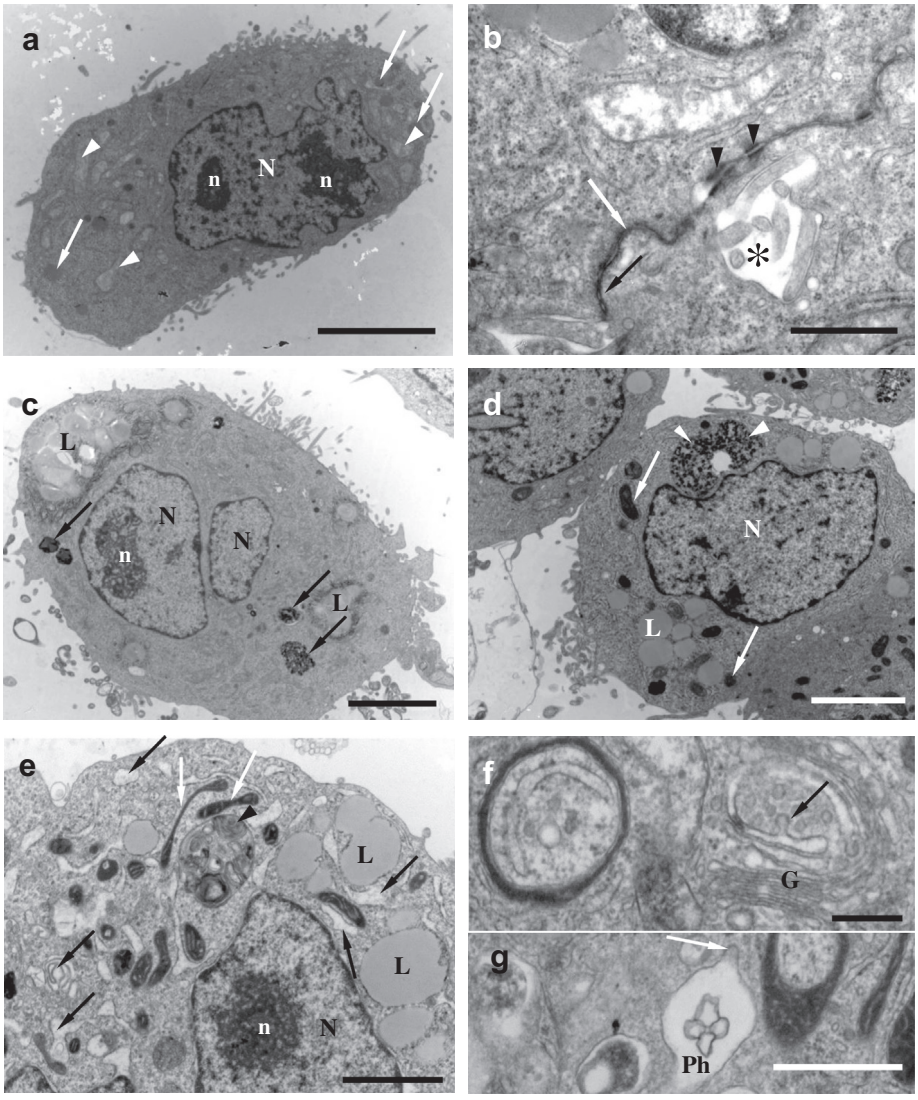

Fig. 9. Ultrastructural changes of HepG2 cells after 24 h of exposure to Cloisite® 30B observed by electron microscopy. Unexposed control cultures (a and b) and HepG2 cells exposed to 22 μg/mL Cloisite® 30B (c), 44 μg/mL Cloisite® 30B (d), and 88 μg/mL Cloisite® 30B (e–g). (a) Unexposed cells with big irregular heterochromatic nuclei (N), prominent nucleoli (n), mitochondria with light matrix (white arrow head) and lipid drops (white arrow). Bar = 5 μm. (b) Differentiated cells exhibiting tight junction (black arrow), adherent junction (white arrow) and desmosomes (black arrow head), showing intracellular spaces (asterix). Bar = 2 μm. (c) Presence of heterophagosomes (black arrow), lipid drops (L) and euchromatic nuclei (N) with visible nucleoli (n). Bar = 1 μm. (d) Cells show heterochromatic nuclei (N), confluent lipid drops (L), heterophagosomes (arrow head) and mitochondria with dense matrix and visible crest (white arrow). Bar = 2 μm. (e) Dilated endomembrane systems (black arrow), mitochondria with dumbbell-shape (white arrow), confluent lipid drops (L), heterophagosomes with membranous debris (black arrow head), euchromatic nuclei (N) and visible nucleoli (n) are observed in the cell. Bar = 2 μm. (f). Dictyosomes (G) producing covered vesicles (black arrow) Bar = 0.5 μm. (g). Endosomes (white arrow) forming phagosomes (Ph). Bar = 0.5 μm.

A lower sensitivity of the hepatic cell line in comparison to the intestinal cell line has been detected. Thus, the EC_{50} (24 h) for the NR assay was 88 μg/mL for HepG2 cells and 50 μg/mL for Caco-2 cells (Gutiérrez-Praena et al., 2011b; Gutiérrez-Praena et al., 2013).

When the same concentration of both clays are compared, for example at 62.5 μg/mL, Cloisite® 30B seems to be slightly more toxic as the percentage of reduced viability found was higher. Thus, the inclusion of modifiers has a role in the toxicity of clays. Other organo-modified clays, such as Cloisite 93A, have also demonstrated to be cytotoxic in HepG2 cells (Lordan et al., 2011). Also, Li et al. (2010) observed a slight decrease in CHO cells viability exposed to 62.5–1000 μg/mL nanosilicate platelets as measured with the MTT and LDH assays.

Considering that Cloisite® 30B showed a high cytotoxicity at the concentrations assayed in comparison to Cloisite® Na+, we focused on that clay to investigate the mechanisms involved in those effects. In the scientific literature different toxic mechanisms such as oxidative stress, inflammation responses, genotoxicity, have been reported to be related to nanoparticles exposure (Bouwmeester et al., 2009). Our results showed a deep decrease of the GSH content in all the concentrations assayed. GSH is the main endogenous antioxidant and is responsible for the maintenance of the intracellular redox balance, detoxification of xenobiotics, and reactive oxygen species (Schafer and Buettner, 2001). GSH depletion could be related with ROS scavenging resulting in the absence of changes in this biomarker. On the other hand, it might be that Cloisite® 30B produces no ROS. In fact, Sharma et al. (2010) also found that Cloisite® Na+ and 30B did not induce ROS production. Other authors, however, observed an increase of intracellular ROS production in HepG2 cells exposed at concentrations of 50 μg/mL Cloisite®Na+ and higher. Conversely, the effect of Cloisite 93A in the same experimental model was less prominent (Lordan et al., 2011).

The results obtained indicate that both clays do not induce inflammatory responses in HepG2 cells. However, Elmore and

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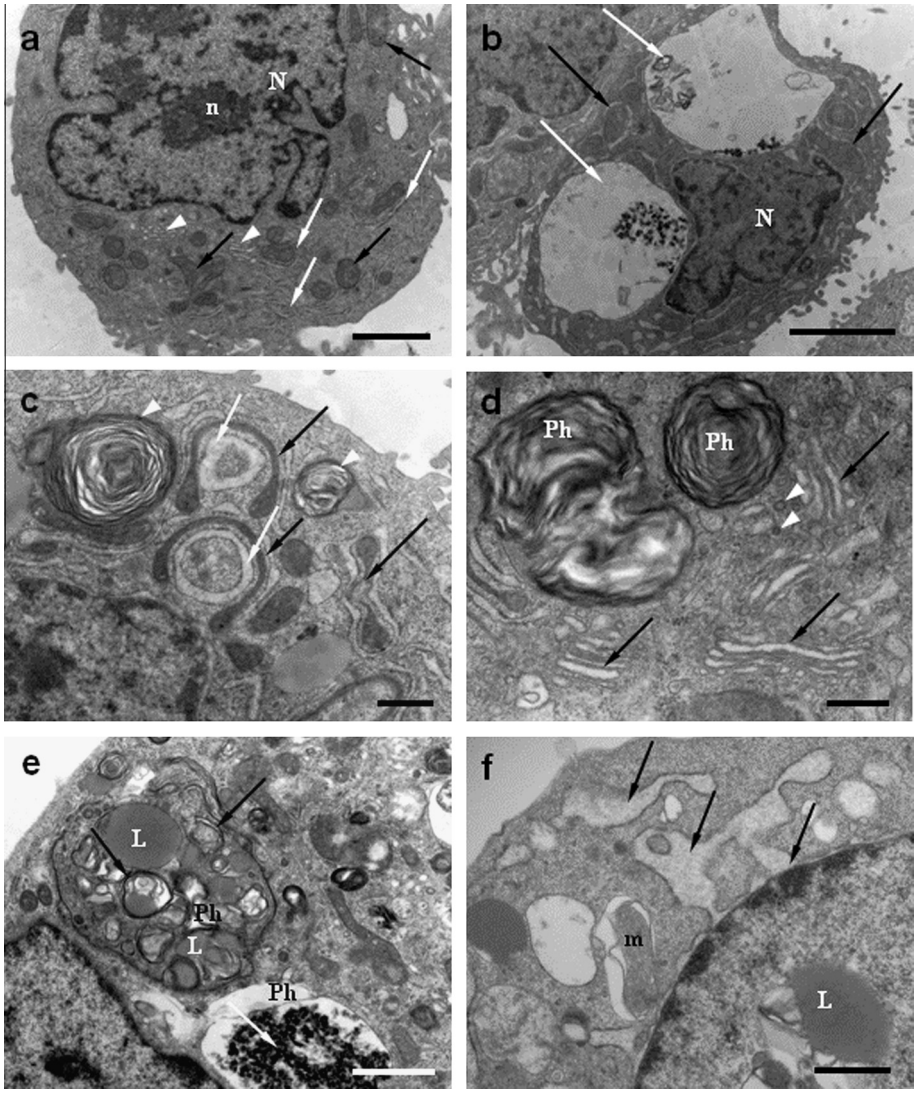

Fig. 10. Ultrastructural changes of HepG2 cells after 48 h of exposure to Cloisite® 30B observed by electron microscopy. HepG2 cells exposed to 22 μg/mL Cloisite® 30B (a), 44 μg/mL Cloisite® 30B (b–d), and 88 μg/mL Cloisite® 30B (e and f). (a) Cells showed nuclei with irregular border and heterochromatin accumulations linked to the inner nuclear membrane (N), nucleoli well developed (n), rough endoplasmic reticulum cisternae (white arrow), dilated dictyosomes (white arrow head) and hypertrophic mitochondria with disorganized crests (black arrow). Bar = 0.5 μm. (b) Cells exhibit chromatin condensation (N), vacuolated cytoplasm with membranous debris inside (white arrow), dilated rough endoplasmic reticulum cisternae associated with hypertrophic mitochondria (black arrow). Bar = 0.5 μm. (c) Dilated rough endoplasmic reticulum cisternae (white arrow) associated with elongated and semicircular mitochondria (black arrow), as well as phagosomes with membranose debris (white arrow head). Bar = 0.2 μm. (d) Phagosomes (Ph), Golgi apparatus with dilated dictyosomes (black arrow) and vesicles covered by clatrine (white arrow head) are observed. Bar = 0.25 μm. (e) Cells show heterophagosomes (Ph) containing membranous debris (black arrow), dense bodies (white arrow) and lipid material (L). Bar = 0.5 μm. (f) Dilated endoplasmic reticulum with big spaces in the nuclear membrane (black arrow), mitochondrial degradation (m) and presence of nuclear lipid inclusions (L) are observed. Bar = 0.5 μm.

Andersen (2003) reported that different clays, including montmorillonite, induced cytotoxicity to several macrophage type cell lines and have hemolytic activity towards several species' red blood cells. Also, it has been reported that montmorillonite clay may promote infection by a direct cytotoxic effect on neutrophils, making them unavailable for bacterial phagocytosis (Dougherty et al., 1985). In relation to genotoxicity, the DNA strand-breaks induced by Cloisite® Na+ were not investigated since no cytotoxic effects were observed in the range of concentrations employed. Li et al. (2010) obtained negative results in the comet assay with CHO cells exposed up to 1000 μg/mL nanosilicate platelets derived from natural montmorillonite. Similarly, Sharma et al. (2010) observed no genotoxic effects induced by unfiltered and filtered Cloisite® Na+ samples. Apart from montmorillonite, other clays such as bentonite and kaolinite have been reported to be not genotoxic or to have a slight effect *in vitro* (Sharma et al., 2000). Cloisite® 30B, however, induced a significant increase in DNA damage after 48 h exposure to 88 μg/mL in our work. This finding agree with Sharma et al. (2010), who found positive results in Caco-2 cells exposed to filtered and unfiltered samples of the organomodified clay from 113 μg/mL. They reported no genotoxicity after 24 h exposure at 85 μg/mL, a concentration similar to the one used in the present study. However, in our case the effect was only observed after a higher exposure time, 48 h. These authors indicated that the quaternary ammonium compound used to modify the clay, was contributing to the genotoxic effects and also that genotoxic effects were not due to oxidative damage. The absence of ROS increase in this study agrees with this hypothesis.

Morphological studies on cell lines exposed to clays are very scarce. In this sense, the present study describes for the first time the ultraestructural changes induced by Cloisite® 30B on hepatic cells. The ultrastructural study constitutes an important key in

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the research of toxic mechanisms of Cloisite® 30B, since it allows exploring the intracellular targets of the clay. The most relevant alterations observed in this study were mitochondrial degeneration, dilated endomembrane systems, heterophagosomes formation, fat droplets appearance and presence of nuclear lipid inclusions. The extent and severity of the cell damage became more widespread as concentration and time after exposure increased. In the present study, cells observed under light microscopy after 48 h of exposure to 88 μg/mL Cloisite® 30B showed a decrease in the growing rate, which indicates cell death. Similar findings were observed by Zia et al. (2011), reporting a concentration-dependent cell rounding and detachment on fibroblast cells exposed to chitin-bentonite clay. Also, bentonite and montmorillonite induced cell lysis on primary neuronal cells after 1 h of incubation; however, no effect was observed on differentiated N1E-115 neuroblastoma cells (Murphy et al., 1993a). Similarly, Murphy et al. (1993b) reported lysis of human umbilical vein endothelial (HUVE) cells exposed to aluminum silicate clays, although undifferentiated N1E-115 neuroblastoma cells and ROC-1 oligodendroglial cells were not lysed by the clays. In addition, in the present study alteration in the lipid metabolism was observed with fat droplets appearance and presence of nuclear lipid inclusions. In this regard, Murphy et al. (1993b) also indicated that all the cells incubated during 6 h with the clays released fatty acids, although it was only significant in the case of HUVE cells, being montmorillonite the clay exerting the highest effects. Moreover, the presence of intranuclear lipid inclusions observed in the present work has been also reported in mouse and rat hepatocytes exposed to other toxicants (Karasaki, 1973; Martino et al., 1981). In our research group, we have previously found the presence of lipid drops in the cytoplasm (Gutiérrez-Praena et al., 2013). However, as far as we know, this is the first work reporting the presence of nuclear lipid inclusions induced by a clay. The intranuclear inclusions appear to be related to abnormalities of membranes of the endoplasmic reticulum and nuclear envelope (Karasaki, 1973). Recently, nuclear lipid droplets have been considered as a domain within the nucleus where nuclear neutral-lipid are stored and organized, which may be involved in nuclear lipid homeostasis (Layerenza et al., 2013). In the present study, the accumulation of lipid drops is related to autophagic processes, since they are frequently found in the heterophagosomes together with membranous debris, and dense bodies. This finding has been also reported in HepG2 cells treated with culture medium without amino acids (Martinet et al., 2006). They state that initiation of autophagy is associated with accumulation of lipid in the cytoplasm. In addition, also the mitochondrial degradation observed in the present work has been related with this cell survival pathway. Hence, regulated changes in mitochondria morphology determine the fate of the cell during autophagy (Gomes et al., 2011). Moreover, the relationship between autophagy and apoptosis is still unclear. Many substances can induces both autophagy and apoptosis in HepG2 cells (Yan et al., 2007; Mei et al., 2011; Xie et al., 2011; Wen et al., 2012). Therefore, autophagy can either cause apoptosis or may be a consequence of apoptosis (Wang et al., 2010).

Studies have been published that used very high doses of micro and nanoparticles for the testing. Unrealistic high dosing can lead to outcomes that may not be related to the inherent toxicity of the material but to the high amount of the material administered (EFSA, 2011). The concentrations used in this study are below the quantity of clay used for nanocomposites in food packaging applications. Making an approximation of what can happen in a real exposure scenario, it is important to know that polymer nanocomposites used as food contact materials contain 4% of clay. Thus, a rigid packaging of 20 g (a small bottle) would contain 0.8 g of clay. The results have shown that 500 μg/mL in a well of a cell culture plate, this is 50 μg Cloisite® 30B, induce a high cytotoxicity. Therefore, if the clay contained in the polymer reaches the consumer, the potential of toxic effects is not negligible. EFSA (2011) considers that in the absence of exposure data, and where it is not possible to determine the nanoform in the food matrix, it should be assumed that all added nanomaterial is present, ingested and absorbed in the nanoform.

5. Conclusions

Our results suggest that caution is necessary as different clays differ in toxicity. The concentrations of Cloisite® Na+ assayed did not show toxicity whereas Cloisite® 30B induced cytotoxicity, GSH decrease, genotoxicity and morphological alterations in HepG2 cells. Further research is needed to determine the potential migration of the clays, to assess the real exposure and to evaluate toxicity with *in vivo* experimental models. Thus, a clearer idea of the food safety of these materials could be derived.

Conflict of Interest

The authors declare that there are no conflicts of interest.

6. Uncited references

Ramadan et al. (2010) and Schreider et al. (1985). Q3

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, the Cell Culture Service and Microscopy Service of Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS), and Remedios García Navarro for providing technical assistance.

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Please cite this article in press as: Maisanaba, S., et al. *In vitro* toxicological assessment of clays for their use in food packaging applications. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.03.043

CAPÍTULO 3 / CHAPTER 3

Sara Maisanaba, Klara Hercog, Metka Filipic, Ángeles Jos, Bojana Zegura

GENOTOXIC POTENTIAL OF MONTMORILLONITE CLAY MINERAL AND ALTERATION IN THE EXPRESSION OF GENES INVOLVED IN TOXICITY MECHANISMS IN THE HUMAN HEPATOMA CELL LINE HEPG2

Enviado a Journal of Hazardous Materials/ Sent to Journal of Hazardous Materials

Elsevier Editorial System(tm) for Journal of Hazardous Materials
Manuscript Draft

Manuscript Number: HAZMAT-D-15-03066

Title: Genotoxic potential of Montmorillonite clay mineral and alteration in the expression of genes involved in toxicity mechanisms in the human hepatoma cell line HepG2

Article Type: Research Paper

Keywords: Cloisite®Na+; toxicity; micronucleus; gene expression; qPCR.

Corresponding Author: Mrs. Sara Maisanaba,

Corresponding Author's Institution:

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Klara Hercog; Metka Filipic; Angeles Jos; Bojana Zegura

Abstract: Montmorillonite, also known as Cloisite®Na+ (CNa+), is a natural clay with a wide range of well-documented and novel applications, such as pharmaceutical products or food packaging. Although considered a low toxic product, the expected increased exposure to CNa+arises concern on the potential consequences on human and environmental health especially as its genotoxicity has scarcely been investigated so far. Thus, we investigated, for the first time, the influence of non-cytotoxic concentrations of CNa+ (15.65, 31.25 and 62.5 μg/mL) on genomic instability of human hepatoma cell line (HepG2) by determining the formation of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) with the Cytokinesis block micronucleus cytome assay. Further on we studied the influence of CNa+ on the expression of several genes involved in toxicity mechanisms using the real-time quantitative PCR. The results showed that CNa+ increased the number of MNi, while the numbers of NBUDs and NPBs were not affected. In addition it deregulated genes in all the groups studied, mainly after longer time of exposure. These findings provide the evidence that CNa+ is potentially genotoxic. Therefore further studies that will elucidate the molecular mechanisms involved in toxic activity of CNa+ are needed for hazard identification and human safety assessment.

Novelty statement

The authors declare that the results included in the present manuscript have not been submitted before to any other scientific journal. To the extent of our knowledge, this is the first study that reports that Cloisite®Na^+ induces alterations in the gene expression as well as genotoxic effects.

Highlights

- Cloisite®Na^+ has a wide range of well-documented and novel applications
- Cloisite®Na^+ induces micronucleus, but not nuclear bridges or nuclear buds in HepG2 cells
- Cloisite®Na^+ induces changes in the gene expression
- Gene alteration is presented mainly after 24h of exposure to Cloisite®Na^+

Sara Maisanaba Hernández
Area of Toxicology. Faculty of Pharmacy. University of Sevilla
C/Profesor García González 2, 41012 Sevilla, Spain
Tel.: +34-954556762; fax: +34-954556422.
E-mail address: saramh@us.es

24th July, 2015

Dear Editor,

We would be very grateful if you consider the manuscript entitled **"Genotoxic potential of Montmorillonite clay mineral and alteration in the expression of genes involved in toxicity mechanisms in the human hepatoma cell line HepG2"** for its publication in "Journal of Hazardous Materials". Although clay minerals have a wide range of applications, in regard to their toxicological profile scarce data are available in the scientific literature about their effects on the genetic material. Thus, to the extent of our knowledge, this is the first work that studies the influence of Montmorillonite (Cloisite®Na^+) on genomic instability by the Cytokinesis block micronucleus cytome assay, as well as, the Cloisite®Na^+ induced modulation of the expression of selected genes involved in the main toxicity mechanisms in HepG2 cell line. The results provide new data about this clay mineral, as it has shown to be potentially genotoxic.

I am looking forward to receiving a positive answer from you.

Sincerely,

Sara Maisanaba Hernández

P.S. Total number of words: 4994

Genotoxic potential of Montmorillonite clay mineral and alteration in the expression of genes involved in toxicity mechanisms in the human hepatoma cell line HepG2

Sara Maisanaba[a*], Klara Hercog[b], Metka Filipic[b], Ángeles Jos[a], Bojana Zegura[b]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[b] National Institute of Biology, Department for Genetic Toxicology and Cancer Biology, Vecna pot 111, 1000 Ljubljana, Slovenia.

*Corresponding author:

Sara Maisanaba

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: saramh@us.es

Tel: +34 954 556762

Abstract

Montmorillonite, also known as Cloisite®Na^+ (CNa^+), is a natural clay with a wide range of well-documented and novel applications, such as pharmaceutical products or food packaging. Although considered a low toxic product, the expected increased exposure to CNa^+arises concern on the potential consequences on human and environmental health especially as its genotoxicity has scarcely been investigated so far. Thus, we investigated, for the first time, the influence of non-cytotoxic concentrations of CNa^+ (15.65, 31.25 and 62.5 µg/mL) on genomic instability of human hepatoma cell line (HepG2) by determining the formation of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) with the Cytokinesis block micronucleus cytome assay. Further on we studied the influence of CNa^+ on the expression of several genes involved in toxicity mechanisms using the real-time quantitative PCR. The results showed that CNa^+ increased the number of MNi, while the numbers of NBUDs and NPBs were not affected. In addition it deregulated genes in all the groups studied, mainly after longer time of exposure. These findings provide the evidence that CNa^+ is potentially genotoxic. Therefore further studies that will elucidate the molecular mechanisms involved in toxic activity of CNa^+ are needed for hazard identification and human safety assessment.

Keywords: Cloisite®Na^+; toxicity; micronucleus; gene expression; qPCR.

1. Introduction

Clays and clay minerals are recognized as the materials of the 21st century because they are abundant, inexpensive and environmentally friendly [1].There are well over 100 documented industrial applications of clay materials. Clays are utilized in the process industries, in agricultural applications, in engineering and construction applications, in environmental remediation, in geology [2], as pharmaceutical and cosmetic products [3], and in many other miscellaneous applications. They belong to the phyllosilicate group that comprises different structurally related compounds [4]. Among them, montmorillonite (Mt) is a smectite, 2:1 phyllosilicate, meaning that it has two tetrahedral sheets sandwiching a central octahedral sheet, and the particles are plate-shaped [5]. It has the following molecular formula: $(Na, Ca)_{0.33}(Al, Mg)_2 (Si_4O_{10}) (OH)_2 \cdot nH_2O$ [6].

Mt, also commercially known as Cloisite®Na^+ (CNa^+), has some of the potential applications mentioned above. For example, it is used as drug delivery system [7] because it is generally considered as a low toxic material compared to other inorganic delivery carriers [8]. Also, in the food industry, it is chemically modified to be incorporated in the food packaging in order to give new materials known as nanocomposites, which represent a new alternative to conventional technologies for improving polymer properties [9].

Taking into account all these well-known and new applications that clay minerals have, the human and environmental exposure to these compounds will probably increase in the near future. Therefore, the potential consequences on human and environmental health are of concern and a safety evaluation of clays is required. Recently the toxic effects and the underlying mechanisms of different clays, including CNa^+, have been reviewed by Maisanaba et al. [10]. Toxic effects of clay minerals in general have been shown to occur mainly after inhalation [5,11], but one of the most likely routes of exposure to these clay minerals for the general population is by oral ingestion, since they are potentially present in food and pharmaceutical products, etc.

Due to the commercial availability of CNa^+ reports on its toxicity are more abundant in the scientific literature compared to others clay minerals. Moreover, due to its wide application and increasing use the interest in toxicological aspects of CNa^+ and its derivatives has increased in the recent years [10]. CNa^+ has been reported to be not toxic in animals [12], however, *in vitro*, CNa^+ has been shown to reduce the proliferation of human normal intestinal cells (INT-

407) and has been cytotoxic at high concentrations [6]. Similarly, Lordan et al., [13] reported cytotoxic activity of CNa^+ in human hepatoma cell line (HepG2), while Maisanaba et al. [14] at lower tested concentrations did not detect cytotoxic activity of CNa^+ in the same cell line as well as in a human colon cell line (Caco-2) [15].

Genotoxicity studies showed that CNa^+ is not mutagenic in *Salmonella typhimurium* [5, 16] and does not induce DNA strand breaks in Caco-2 cells [5]. *In vitro* data on the induction of reactive oxygen species (ROS) by CNa^+ are contradictory. Some literature data report that CNa^+ induces ROS formation [13], while at lower concentrations no oxidative stress has been detected [5]. Although these scarce data point out that CNa^+ is not genotoxic there is a need for further research on its genotoxic potential to obtain information that will enable the elucidation of the underlying mechanisms of its action at the molecular level. Therefore, the aim of our study was to evaluate whether CNa^+ had influence on genomic instability by analyzing the formation of micronuclei (MNi), nuclear buds (NBUD), and nucleoplasmic bridges (NPB) in the human hepatoma cell line (HepG2 cells) by the Cytokinesis block micronucleus cytome assay (CBMN). Moreover, the CNa^+ induced modulation of the expression of selected genes involved in the metabolism, immediate-early response/signaling, DNA damage response, oxidative stress and programmed cell death was investigated by real-time quantitative PCR (qRT-PCR).

2. Materials and Methods

2.1. Chemicals

Minimum essential medium (MEM), TRIzol® reagent and B27 supplement were from Gibco BRL (Paisley, Scotland); foetal bovine serum (FBS), non-essential aminoacids (NEAA), penicillin/streptomycin, L-glutamine and phosphate buffered saline (PBS) from PAA (Pasching, Austria). Cytochalasin B (Cyt-B), acridine orange (AO), dimethyl sulfoxide (DMSO [CAS 67-68-5]), trypsin, etoposide (ET) [CAS 33419-42-0] and benzo(*a*)pyrene (B(*a*)P) [CAS 50-32-8], were obtained from Sigma–Aldrich (St. Louis, USA). High Capacity cDNA Archive Kit and Taqman Gene Expression Assays were from Applied Biosystems (Forest City, CA, USA), TaqMan Universal PCR Master Mix from Applied Biosystems, (Branchburg, NJ, USA) and Human GAPDH from Applied Biosystems (Warrington, UK).

Cloisite® Na^+ was obtained from Southern Clay Products, INC (Austin, Texas, USA). The clay was characterized by thermogravimetric analysis (TGA), X-ray dif- 115 fraction (XRD) and particle size distribution (PSD) as described in Maisanaba et al. [14].

2.2. Clay test solutions

The test concentrations for the clay were selected according to previous dispersion experiments in order to avoid interferences with the measurement system [14]. The highest concentration tested was 62.5 µg/mL. Test solutions were prepared in serum-free medium supplemented with B27. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

2.3. Cell culture and treatment

HepG2 (human hepatocellular carcinoma epithelial cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in a monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 2% B27 (Gibco, New Zealand), 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin/ streptomycin (Gibco, New Zealand). Cells were grown at 37°C and 5% CO_2 in humidified atmosphere. Cells were used at passages between 12 and 18.

Prior to the treatment, the cells were seeded onto 96-well plates (4000 cells/well) to evaluate the cytotoxicity with the MTS assay, 6-well tissue culture treated plates (400 000 cells/ well) for the CBMN assay and 25 cm^2 culture flask (800 000 cells/ plate) (all Corning Costar Corporation, New York, USA) for qRT-PCR gene expression analysis, and incubated for 24h to attach. The medium was then replaced with fresh medium containing graded concentrations of CNa^+ and incubated for 4 and 24h for the MTS assay and qRT-PCR gene expression analysis and 24h for the CBMN test. The cytotoxicity of the clay was determined at 6.25, and 62.5 µg/mL. For the CBMN test and gene expression analyses the cells were exposed to non-cytotoxic concentrations, 15.65, 31.25, 62.5 µg/mL and 6.25, 62.5 µg/mL, respectively. B(*a*)P (2.5 µM in CBMN and 30 µM in q-PCR), a well established pro-carcinogen that needs metabolic activation was used as the positive control. In the CBMN assay an additional positive control, etoposide (ET; 1µg/mL), was used.

2.4. Cytotoxicity assay

Cytotoxicity of CNa^+ was determined with the MTS assay (CellTiter96 AQueous Non Radioactive Cell Proliferation Assay; Promega, Madison, USA) according to the manufacturer's

instructions. Cell viability was determined by comparing the optical density of the wells containing the cells treated with the clay with those of the control group. Two independent experiments were performed, each in five replicates per treatment point. Student's t test was used in order to evaluate significant differences.

2.5. Cytokinesis block micronucleus assay (CBMN)

After the 24-h treatment, the cells were washed twice with 1x PBS buffer, the medium containing Cyt-B (2 μg/ml) was added and the cells were incubated for additional 26 h at 37°C and 5% CO_2 in humidified atmosphere. At the end of the exposure floating and adherent cells were collected by trypsinization. Subsequently the cells were incubated in a cold hypotonic solution (75 mM KCl) for 5 min and fixed with a mix of methanol/acetic (3:1; v/v) and formaldehyde. The fixation was repeated three times and the fixed cells were put on microscope slides and air dried. The slides were stained with acridine orange (10 μg/ml) and examined under the fluorescence microscope (Olympus BX61, Japan). Micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBs) were counted in 1000 binucleated cells (BNC) per experimental point at 400x magnification according to the criteria published by Fenech [17,18]. The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula [M1+ 2M2 + 3(M3 +M4)]/500, where M1, M2, M3 and M4 represent the number of cells with one to four nuclei, respectively. The experiments were repeated three times independently. Chi-square test was used to compare the number of MNi, NBUDs, or NPBs between the control group and CNa^+ exposed cells; $P < 0.05$ (*) and $P < 0.01$ (**) was considered as statistically significant.

2.6. Real-time quantitative PCR (QRT-PCR) analysis

After the incubation, cells were washed twice with 1x PBS and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications described in Štraser et al. [19]. Briefly, glycogen (20 μg/mL) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at -20°C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water. Three independent experiments were performed. The RNA was transcribed to cDNA using 1 μg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. Gene expression was quantified by RT-qPCR using TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays: *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1),

Hs01054797_g1; *CYP1A2* (cytochrome P450, family 1, subfamily A, polypeptide 2), Hs00167927_m1; *CYP3A4* (cytochrome P450, family 3, subfamily A, polypeptide 4) Hs00604506_m1; *GSTA1* (glutathione S-transferase alpha 1), Hs00275575_m1; *UGT1A1* (UDP glucuronosyltransferase 1 family, polypeptide A1), Hs02511055_s1; *FOS* (FBJ murine osteosarcoma viral oncogene homolog), Hs04194186_s1; *JUNB* (jun B proto-oncogene) Hs00357891_s1; *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) Hs00153408_m1; *TGFB2* (transforming growth factor, beta 2) Hs00234244_m1; *CDKN1A* (cyclin-dependent kinase inhibitor 1A') Hs00355782_m1; *CHEK1* (checkpoint kinase 1) Hs00967506_m1; *ERCC4* (excision repair cross-complementing rodent repair deficiency, complementation group 4) Hs00193342_m1; *GADD45A* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; *MDM2* (Mdm2, 'MDM2 oncogene, E3 ubiquitin protein ligase), Hs01066930_m1; *TP53* (tumor protein P53), Hs01034249_m1; *BAX* (BCL2-associated X protein), Hs00180269_m1; *BCL2* (B-cell CLL/lymphoma 2), Hs00608023_m1; *CASP3* (caspase 3, apoptosis-related cysteine peptidase), Hs00234387_m1; *CASP8* (caspase 8, apoptosis-related cysteine peptidase), Hs01018151_m1; *CASP9* (caspase 9, apoptosis-related cysteine peptidase), Hs00609647_m1; *FAS* (Fas cell surface death receptor), Hs00236330_m1; *CAT* (catalase), Hs00156308_m1; *GCLC* (glutamate-cysteine ligase, catalytic subunit), Hs00155249_m1; *GPX1* (glutathione peroxidase 1), Hs00829989_gH; *GSR* (glutathione reductase), Hs00167317_m1; *SOD1A* (superoxide dismutase 1), Hs00533490_m1. Amplification of *GAPDH* probe (Human Endogenous Controls, Cat. No.: 4310884E, Applied Biosystems, USA) was performed as an internal control. The conditions for the PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using an accurate quantification taking into account a control pattern (10x, 100x, 1000x, 10000x) and a normalization with the control group. The expression levels of target mRNAs were normalized to the GAPDH mRNA level. Two independent experiments were performed each time in triplicates. Statistical significance between treated groups and controls was determined by two tailed Student's t test, and $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) was considered significant with respect the control group. An up-regulation and down-regulation of ≥ 1.5-fold change and ≤ 0.7, respectively, compared to control was considered a positive response.

3. Results

3.1. Viability of HepG2 exposed to CNa^+

No significant decrease of cell viability was observed after CNa^+ (6.25 and 62.5 μg/mL) treatment for 4 and 24h (data not shown); therefore, these concentrations were used for further experiments.

3.2. Genotoxicity of CNa^+ in HepG2: Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of CNa^+ in HepG2 cells was evaluated using the CBMN assay (Fig. 1). Following 24h exposure, CNa^+ induced a statistically significant increase only in the frequency of MNi (2.8-fold more) in BNCs at the highest concentration tested (62.5 μg/mL) (P<0.0001), as well as, significant differences in MNed cells (P<0.001), being about 2.6-fold higher than in the control group, while no induction of NPBs and NBUDs was observed. At lower concentrations used (15.65 and 31.25 μg/mL) no changes in the frequency of MNi, NPBs or NBUDs compared to non-treated control were determined. The positive controls B(a)P (2.5 μM) and ET (1 μg/mL) induced statistically significant 2.5-fold and 3.7-fold increases in the frequency of MNed cells and 2.6-fold and 4.2-fold increases in the frequency of MNi, respectively (Fig.2). CNa^+ did not affect division of cells, while positive controls decreased NDI in a statistically significant way (Fig.3).

3.3. Effect of CNa^+ on mRNA expression

The mRNa expression of a wide range of selected genes was analyzed after 4 and 24h of exposure to 6.25 and 62.5 μg/mL of CNa^+ by quantitative real-time PCR (Table 1). The values presented in the Table 1 represent relative increase of gene expression of the selected genes compared to control group (untreated cells).

3.3.1. Effect of CNa^+ on mRNA expression of genes involved in metabolism

In HepG2 cells exposed to CNa^+ for 4h, the mRNA levels of the genes assayed did not show any change compared to the control group. However, after 24h of exposure, four out of five genes tested (*CYP1A1, CYP1A2, CYP3A4* and *GST1A*) were more than 1.7-fold up-regulated at applied concentrations. On the other hand, *UGT1A1* presented a significant (P<0.01) down-regulation (0.69±0.04) at 62.5 μg/mL CNa^+.

3.3.2. Effect of CNa^+ on mRNA expression of immediate –early response/signaling genes

After the exposure of HepG2 cells to CNa^+ for 4h, the mRNA expressions of *FOS*, *MYC* and *TGFB2* were not affected, while after 24h the expression of these genes was up-regulated (≥ 1.5 fold). *FOS* and *TGFB2* at both concentrations tested, while for *MYC* only at the highest. The expression of *JUNB* was significantly down-regulated at 62.5 µg/mL CNa^+ after 4h exposure, while after 24h CNa^+ treatment *JUNB* mRNA level increased dose dependently.

3.3.3. Effect of CNa^+ on mRNA expression of DNA damage responsive genes

After 4h exposure to CNa^+ no changes in the expression of *TP53* and its downstream-regulated genes *MDM2, CDKN1A, GADD45A, CHEK1* and *ERCC4* were determined. Following 24h exposure DNA damage responsive genes *CDKN1A (*approximately 1.5-fold increase at both concentrations) and *GADD45A* (2-fold and 4-fold at 6.25 and 62.5 µg/mL, respectively) were significantly up-regulated, while other DNA damage responsive genes studied were not affected by CNa^+.

3.3.4. Effect of CNa^+ on mRNA expression of oxidative stress responsive genes

In cells exposed to CNa^+ for 4h, the mRNA levels of the genes selected did not show significant changes. After 24h, only *CAT* presented an important down-regulation at both concentrations used (0.67 and 0.52-fold, respectively), and *GCLC* showed a significant 1.92-fold up-regulation at 62.5 µg/mL. Other oxidative stress induced genes (*GPX1, GSR* and *SOD1A*) included were not affected under the conditions applied in the study.

3.3.5. Effect of CNa^+ on mRNA expression of apoptosis responsive genes

No changes in the expressions of selected apoptosis/survival responsive genes were observed in HepG2 cells exposed to CNa^+ for 4h. After 24h exposure only the expression of anti-apoptotic gene *BCL2* (approximately 2-fold increase) at both concentrations assayed, and *CASP3* (1.5-fold increase) at the highest concentration used, displayed significant up-regulation compared to the control group. The expression of pro-apoptotic genes *BAX, CASP8* and *CASP9* were not altered by CNa^+ after 24h exposure.

4. Discussion

In spite of the wide number of applications that clay minerals have, their toxic potential has scarcely been investigated so far. Previous reports have indicated that CNa^+ is not genotoxic in different systems like *Salmonella typhimurium* in the Ames test [5, 16] and the Comet Assay in Caco-2 cells [5]. To our knowledge this is the first study to show that CNa^+ can affect the genomic instability of HepG2 cells determined with CBMN assay. This method enables simultaneous detection of multiple parameters associated with chromosomal instability: micronuclei (MNi) induction that reflect structural and numerical chromosomal aberration, nucleoplasmic bridges (NPB) formation that are an indicator of chromosomal rearrangements, DNA miss-repair, or telomere end-fusions, and nuclear buds (NPBs) that are a marker of gene amplification [17]. CNa^+ increased the frequency of micronuclei but there was no influence of the formation of NPBs and NBUDs in HepG2 cells.

Till now the genotoxicity of $CNa^+$was studied only with the comet assay in human colon (Caco-2) and hepatic (HepG2) cancer cell lines. The results showed that CNa^+ (up to the concentration 170 μg/mL) did not induce DNA strand breaks after 24 h of exposure in Caco-2 cells. Similarly, CNa^+ was not genotoxic in HepG2 [20]. However, genotoxicity data of quaternary ammonium salt modified clays based on CNa^+ showed a different profile. Thus, Cloisite®20A and Clay1 did not induce changes in both cell lines [21], whereas Cloisite®30B and Clay2, induced DNA damage under the same conditions of the exposure [5, 21].

Toxicogenomic analyses are becoming widely used as a tool for hazard identification and risk assessment of genotoxic properties of different substances; therefore, we further elucidated the mechanisms of CNa^+ toxic activity by measuring the changes in the expression of the genes involved in different key pathways. To the best of our knowledge this is the first study where the influence of CNa^+ on the mRNA level was researched. Until now there are no literature data on the biotransformation and detoxification of CNa^+. In the present study CNa^+ increased the mRNA level of studied CYP450 isoforms (*CYP1A1, CYP1A2, CYP3A4*) that are highly inducible phase I enzymes and are activators of numerous xenobiotic compounds. For the first time the involvement of CYPs as a response to toxicity of CNa^+ was shown. Moreover, the level of mRNA of glutathione S-transferase (*GST1A1*) was increased. Glutatione-S-transferases (GSTs) are principal phase II enzymes in metabolic detoxification process and play a significant role in the detoxification of electrophiles by glutation conjugation [22]. Another very important family of phase II enzymes is UDP-glucuronosyltransferases (UGTs) that catalyze the glucuronidation of endogenous and exogenous compounds (reviewed in Oda et al. [23]). CNa^+ significantly down-regulated the expression of *UGT1A1* in HepG2 cells. In general,

conjugation with phase II enzymes is considered the detoxification phase of xenobiotic metabolism, although in certain situations, it could result in activated metabolites and increase in toxicity [24].

Some of the immediate early response genes exhibit a very rapid and significant enhancement of their mRNA levels when liver cells are induced to proliferate. These genes include many proto-oncogenes, such as *c-MYC, c-FOS, c-JUN, JUNB, JUND* [25] and their deregulation contributes to the genesis of most human tumours [26]. In HepG2 cells all studied genes involved in signaling and immediate-early response (*FOS, JUNB, MYC, TGFB2*) have shown strong up-regulation after 24 hours of exposure to CNa^+. The induction of *FOS* is known to be indicative for DNA-damage and is increased by a wide variety of DNA-damaging agents [27], while *MYC* proto-oncogene regulates many biological functions, including cell growth, proliferation, apoptosis, differentiation, and transformation [28]. *JUNB* is a key cell cycle regulator able to arrest the G1/S phase transition through transcriptional inhibition of Cyclin D1 [29]. TGFB2, a potent anti-inflamatory cytokine, belongs to the transforming growth factor Bs (TGF-Bs) that regulate a wide variety of cellular processes, and were shown to inhibit epithelial cell proliferation by delaying or arresting progression through the late portion of G1 [30]. The results of the present study show that CNa^+ influences cell signaling processes that determine the faith of the cell that can be either cell-cycle arrest, proliferation, apoptosis, or DNA damage repair.

The tumor-suppressor gene, *TP53*, plays the central role in the cellular response to agents or conditions that damage DNA by activating the transcription of several essential genes controlling cell cycle arrest/DNA repair, senescence, differentiation and apoptosis [31]. In the present study we did not detect any changes in the expression of *TP53*. This is not unusual, as it is known that DNA damage activates the p53 protein predominantly through its phosphorylation by DNA damage- responsive kinases and, to lesser extent, through up-regulation of gene expression [32]. After 24 h exposure *TP53* downstream regulated genes, *CDKN1A* and *GADD45A*, were up-regulated, which can be associated with the cell cycle arrest due to DNA damage. Cyclin-dependent kinase inhibitor 1A (*CDKN1A*), is an important CDKI that induces cell cycle arrest, inhibits cell proliferation and is directly involved in DNA repair, including nucleotide excision repair (NER) [33]. The role of *GADD45A* is to control the cell cycle G2-M checkpoint, the DNA repair process and apoptosis [34]. On the contrary, the gene expression of checkpoint kinase 1 (*CHEK1*), a Ser/Thr kinase that is involved in mediating the cellular response to DNA-damage (reviewed in Oza et al. [35]), and *ERCC4* that is involved in

NER, as well as *MDM2* that enhances the tumorigenic potential of the cells and promotes cell survival and cell cycle progression [36], were not affected by CNa^+.

To date, there are no published reports concerning possible changes in the expression of genes involved in oxidative stress in cells exposed to CNa^+. Therefore in the present study we measured the expression of the most important antioxidant enzymes at the mRNA level. As already mentioned above CNa^+ increased the mRNA level of *GST1A1*, a member of glutathione S-transferase (*GST*) family, which participate in a detoxification pathway that acts via the conjugation of the substance with glutathione (GSH). GSH plays a central role in intracellular antioxidant defense. It can exist in either a reduced (GSH) or oxidized (GSSG) form. Under steady state conditions, cells maintain a resting level of GSH/GSSG know as the redox state [37]. Two enzymes are involved in maintaining cellular redox, namely GPX that catalysis the reduction of peroxides and the formation of GSSG and GSR that reduces the GSSG back to GSH [38]. As a response to oxidative stress GSH can be depleted in cells and is then consequently synthesized de novo in a so called γ-glutamyl cycle that requires two ATP dependent enzymes, glutamate-cysteine ligase (GCLC) and glutathione synthase (GS) [39]. The up-regulation of the gene coding for *GCLC* in HepG2 cells exposed to CNa^+, indicated possible response of cells to a depletion of GSH and its biosynthesis due to the toxic effects of CNa^+. The enzymes involved in maintaining cellular redox, *GPX1* and *GSR,* were not affected at the transcriptional level by CNa^+ at none of the exposed times and concentrations. Furthermore, we explored the gene expression of the primary antioxidant enzymes in cells, superoxide dismutase (SOD) and catalase (CAT) that are more important in the protection of cells than the glutathione redox cycle when severe oxidative stress occurs [40]. The mRNA level of *SOD1A* was not changed, while *CAT* was significantly dose dependently down-regulated after 24h exposure of HepG2 cell to CNa^+.

There is only limited data describing the induction of oxidative stress by CNa^+. In HepG2 cells CNa^+ at relatively high concentrations ranging from 50-1000 µg/ml increased the formation of reactive oxygen species (ROS), which coincided with increased cell membrane damage [13]. On the contrary Sharma et al. [5] did not detect induction of ROS in Caco-2 cells at concentrations up to 170 µg/ml. A subchronic *in vivo* study on rats that were orally exposed to Clay1 (40 mg/kg/day in the diet), a Mt modified with a quaternary ammonium salt for 90 days, showed in the liver no induction of oxidative stress markers such as GSH/GSSG levels and their ratio [41], lipid peroxidation, and the activities and protein content of antioxidative enzymes such as SOD, CAT, GPX and GST. In addition no changes in expression of SOD and CAT

at the gene and protein level determined with the qRT-PCR and Western blot, respectively, were confirmed [41].

The most common form of cell death that plays a pivotal role in the maintenance of tissue homeostasis is apoptosis, programmed cell death. The major apoptotic pathways include the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [42]. In this respect we measured the expression of several genes involved in the apoptotic processes. In the present study we showed that exposure of HepG2 cells to CNa^+ induced significant transcriptional changes of anti-apoptoptic gene *BCL2*, while the expression of pro-apoptotic gene *BAX* was not affected. From all caspase genes included in our study only *CASP3* was up-regulated by CNa^+, while the mRNA levels of *CASP9* and *CASP8* were not changed. Caspases are proteases that act as essential initiators and executioners of the apoptotic process. Classically, the caspase cascade is initiated via cleavage of the so-called initiator caspases (-2, -8, -9, and -10), most likely by autoproteolysis. Initiator caspases, in turn, cleave and activate the executioner caspases (-3, -6, and -7) [43]. This results in mediation and amplification of the death signal and eventually cell death. The results of gene deregulations of pro- and anti-apoptotic genes do not allow for clear interpretation whether CNa^+ induced apoptosis or not. The up-regulation of *BCL2* suggests that apoptosis is suppressed; while on the other hand the up-regulation of *CASP3* indicates potential involvement of intrinsic pathway of apoptosis in CNa^+ toxicity. As the obtained results are contradictory the mechanisms involved in cell death induced by CNa^+ need to be further elucidated.

Regarding to programmed cell death, other authors such as Lordan et al. [13] reported that after 24 h, no increase in caspase-3/7 activity was detected in HepG2 cells treated with CNa^+ and Cloisite 93A, an organically modified Mt. Similarly also Maisanaba et al. [14] reported no induction of apoptosis with the commercial CNa^+-based clay Cloisite®30B in the same cell type. In another study on HepG2 cells, CNa^+ statistically significantly increased the activity of caspase 3/7 after 4.5h (at 500 µg/mL) and 48h (33-500 µg/mL) of exposure [44]. On the contrary, it induced apoptosis in SK-MEL 28 human melanoma cells only after 48 hours of exposure at 33 µg/mL and higher [44]. Moreover, Liu et al. [12] observed an increase in the caspase 3 activity in human embryonic kidney (HEK) 293 cells treated with 1 g/L CNa^+, whereas with the modified oligo(styrene-co-acrylonitrile)-montmorillonite (PSAN-MMT) the increase was lower compared to CNa^+. In addition PSAN-MMT and CNa^+ influence on p53 mRNA and protein level showed higher effect of CNa^+ clay in HEK 293 cells.

5. Conclusions

Based on the results of the present study we can conclude that CNa^+ is potentially genotoxic as it induced the formation of micronuclei at non-cytotoxic concentrations and can therefore represent a risk for human health especially when considering long term exposure. The changes of the expression of several studied genes provide new insights into the mechanisms of CNa^+ potential toxicity. Nevertheless, there is still a need to further confirm these results on the protein level, which will help to clarify the mechanisms involved in CNa^+ genotoxic activity.

Acknowledgements

The authors wish to thank Junta de Andalucía (AGR5969) and Slovenian Research Agency (ProgramP1-0245) for the financial support and to the Technological Institute of Packaging, Transport, and Logistics (ITENE) for the donation of the clay. Sara Maisanaba also gratefully acknowledges Junta de Andalucia for her predoctoral grant associated to the AGR5969 project.

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Figure captions

Figure 1. Flourescent micrographs of HepG2 cells exposed to CNa^+ (62.5 µg/ml for 24 h) **a**) binucleated cell, **b)** binucleated cell with MN, **c)** binucleated cell with two MNi, **d)** binucleated cells with NBUD, **e)** binucleated cells with NBUD and MN and **f)** binucleated cell with NBP.

Figure 2. Number of binucleated cells with **a**) micronucleated cells (MNed cells), **b**) micronuclei (MNi), **c**) nuclear bridges (NPBs) and **d**) cells with nuclear buds (NBUDs) per 1000 binucleated HepG2 cells after the exposure to CNa^+ (0, 15.65, 31.25 and 62.5 µg/mL) for 24 h. The number of MN cells shows how many cells contained MN, while MNi shows the overall number of MN as some cells can possess more than one MN. Significant differences between CNa^+ treated cells and the control group (0) is indicated by $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$, and $****P<0.0001$. Benzo(*a*)pyrene (2.5 µM) and Etoposide (1 µg/mL) were used as the positive controls.

Figure 3. The influence of CNa^+ (0, 15.65, 31.25 and 62.5 µg/mL) on the nuclear division index (NDI) after 24h exposure. Significant differences between CNa^+-treated cells and the control group (0) is indicated by $*P < 0.05$ and $**P < 0.01$. Benzo(*a*)pyrene (2.5 µM) and Etoposide (1 µg/mL) were used as the positive controls.

Table caption

Table 1. Effect of CNa^+ on expression of mRNAs of selected genes involved in metabolism, inmediate-early response/signaling, DNA damage, apoptosis/survival and oxidative stress responses in HepG2 cells.

Table

Mechanisms involved	Gene symbol	CNa^+ (µg/mL)	4h	24h	Entrez gene name
			Mean±SD	Mean±SD	
Metabolism (activation/detoxification)	***CYP1A1***	6.25	0.95±0.07	**1.70±0.13***	Cytochrome P450 family 1. Subfamily A. polypeptide 1
		62.5	0.95±0.05	**1.72±0.3**	
		B(a)P 30µM	**1.91±0.43**	**130.32±9.22****	
	CYP1A2	6.25	0.80±0.15	**2.60±2.45**	Cytochrome P450 family 1. Subfamily A. polypeptide 2
		62.5	1.19±0.18	**1.82±0.78**	
		B(a)P 30µM	**1.76±0.73**	**79.78±15.01***	
	CYP3A4	6.25	0.91±0.24	**2.90±1.16**	Cytochrome P450 family 3. Subfamily A. polypeptide 4
		62.5	0.80±0.33	**4.06±1.84**	
		B(a)P 30µM	1.11±0.17	**2.66±0.18****	
	GST1A1	6.25	0.80±0.15	**1.88±0.34**	Glutathione S-transferase alpha 1
		62.5	0.85±0.07	**1.90±0.48**	
		B(a)P 30µM	0.75±0.01***	**0.60±0.25**	
	UGT1A1	6.25	0.92±0.13	0.84±0.26	UDP glucuronosyltransferase 1 family. polypeptide A1
		62.5	0.85±0.23	**0.69±0.04****	
		B(a)P 30µM	0.97±0.02	**5.57±0.96***	
Immediate-early response/signaling	***FOS***	6.25	1.05±0.12	**10.35±2.32***	FBJ murine osteosarcoma via oncogene homolog B
		62.5	0.94±0.68	**20.38±6.74**	
		B(a)P 30µM	1.23±0.73	**12.49±5.54**	
	JUNB	6.25	0.94±0.01**	**1.77±0.35**	Jun B proto-oncogen
		62.5	**0.63±0.09***	**2.76±0.02*****	
		B(a)P 30µM	1.13±0.36	**2.41±0.46***	
	MYC	6.25	1.06±0.08	1.29±0.14	V-Myc avian myelocytomatosis viral oncogene homolog
		62.5	1.00±0.06	**1.66±0.32**	
		B(a)P 30µM	1.07±0.07	**0.78±0.06***	
	TGFB2	6.25	1.26±0.34	**4.04±1.05**	Transforming growth factor. beta 2
		62.5	1.07±0.7	**2.94±0.62***	
		B(a)P 30µM	0.10±0.77	**0.36±0.09***	

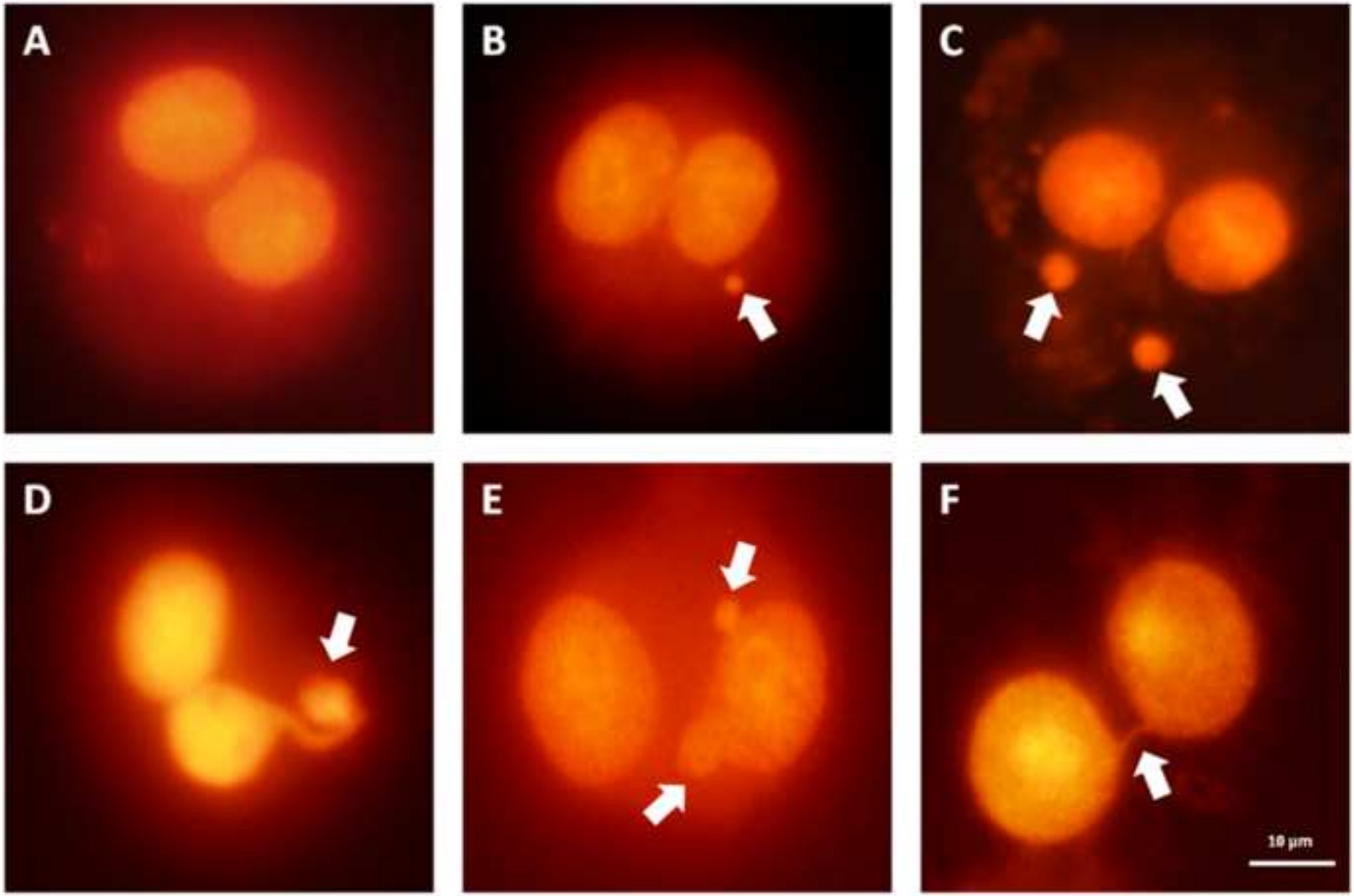
DNA damage responsive	***TP53***	6.25	1.01±0.1	1.12±0.08	Tumor protein p53
		62.5	1.04±0.01*	0.92±0.04	
		B(a)P 30µM	0.10±0.03	1.23±0.16	
	MDM2	6.25	1.08±0.01**	1.15±0.07	Oncogene, E3 Ubiquitin Protein Ligase
		62.5	1.05±0.02	1.15±0.01**	
		B(a)P 30µM	0.10±0.03	1.13±0.21	
	CDKN1A	6.25	1.04±0.02	**1.49±0.002**	Cyclin-dependent kinase inhibitor 1A (p21.Cip 1)
		62.5	1.00±0.02	**1.48±0.07****	
		B(a)P 30µM	1.11±0.08	**7.33±2.02***	
	GADD45A	6.25	1.08±0.11	**1.92±0.59**	Growth arrest and DNA-damage-inducible. alpha
		62.5	1.08±0.05	**3.91±0.77***	
		B(a)P 30µM	1.05±0.24	**2.81±0.31***	
	CHEK1	6.25	0.99±0.06	1.03±0.28	Checkpoint kinase 1
		62.5	0.94±0.06	0.99±0.24	
		B(a)P 30µM	1.06±0.02	**0.7±0.01*****	
	ERCC4	6.25	1.00±0.02	1.09±0.05	Excision repair cross-complementing rodent repair deficiency. complementation group 4
		62.5	1.01±0.13	1.04±0.05	
		B(a)P 30µM	1.06±0.04	**1.68±0.31**	
Oxidative stress	***GCLC***	6.25	0.91±0.2	1.38±0.12*	Glutamate-cysteine ligase. catalytic subunit
		62.5	0.88±0.18	**1.92±0.23***	
		B(a)P 30µM	0.96±0.16	**2.14±0.21***	
	GPX1	6.25	0.81±0.12	1.03±0.29	Glutathione peroxidase 1
		62.5	0.75±0.04	1.04±0.2	
		B(a)P 30µM	0.73±0.01	**1.67±0.19**	
	GSR	6.25	0.83±0.16	1.10±0.13	Glutathione reductase
		62.5	0.86±0.07	1.11±0.07	
		B(a)P 30µM	0.82±0.15	1.43±0.24	

	SOD1A	6.25	0.81±0.16	1.14±0.35	Superoxide dismutase 1
		62.5	0.77±0.03**	1.02±0.21	
		B(a)P 30µM	0.77±0.001***	1.15±0.21	
	CAT	6.25	1.00±0.07	**0.67±0.29**	Catalase
		62.5	0.94±0.11	**0.52±0.26**	
		B(a)P 30µM	0.88±0.07	**0.43±0.02*** **	
Apoptosis/survival	***BAX***	6.25	0.97±0.04	1.17±0.24	Apoptosis regulator BAX. BCL2 associated X protein
		62.5	0.97±0.11	0.81±0.02	
		B(a)P 30µM	0.97±0.16	1.35±0.05	
	BCL2	6.25	0.9±0.08	**1.90±0.24**	B-cell CLL/lymphoma 2
		62.5	0.95±0.06	**1.88±0.02*** **	
		B(a)P 30µM	1.05±0.11	0.10±0.05*	
	CASP3	6.25	1.00±0.09	1.12±0.06	Caspase 3 apoptosis-related cysteine peptidase
		62.5	0.92±0.08	**1.55±0.03** **	
		B(a)P 30µM	1.13±0.1	1.03±0.17	
	CASP8	6.25	1.05±0.09	1.26±0.14	Caspase 8 apoptosis-related cysteine peptidase
		62.5	0.99±0.11	1.32±0.1*	
		B(a)P 30µM	1.05±0.11	**2.11±0.14** **	
	CASP9	6.25	1.06±0.09	1.07±0.07	Caspase 9 apoptosis-related cysteine peptidase
		62.5	1.03±0.06	0.93±0.02	
		B(a)P 30µM	1.03±0.08	0.9±0.06	
	FAS	6.25	0.97±0.04	1.20±0.2	Fas (TNF receptor superfamily. member 6).
		62.5	0.99±0.12	1.30±0.09*	
		B(a)P 30µM	1.15±0.07	**4.86±0.3** **	

Table 1. The results are expressed as relative mRNA expression normalized to the control group. Data are means ± SD of three independent experiments. Significant differences between CNa^{+} treated cells and the control group is indicated by *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (One way ANOVA with Dunnett´s test post test). Bold values indicate up or down-regulation of genes.

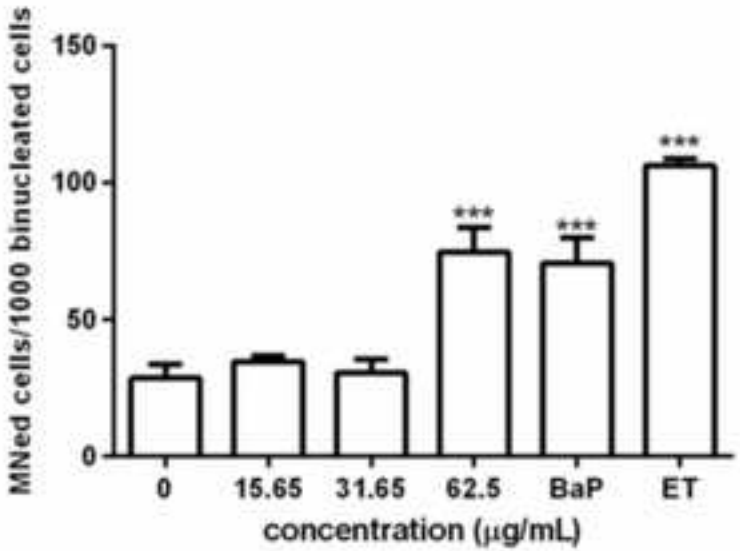
Figure

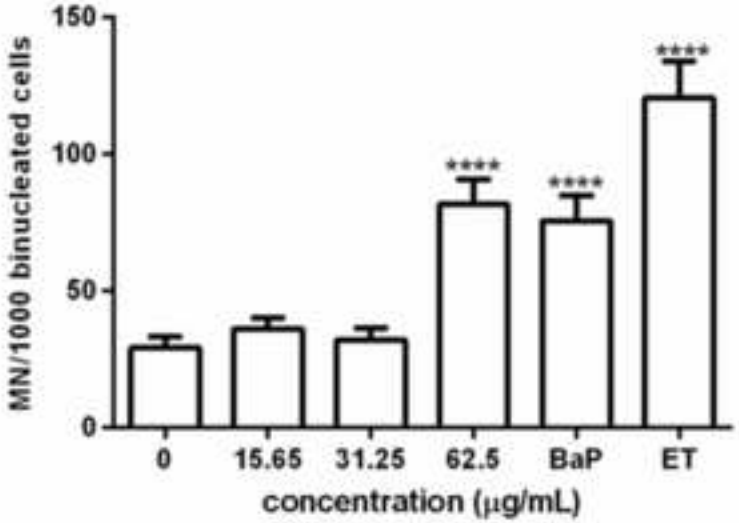
Figure 1

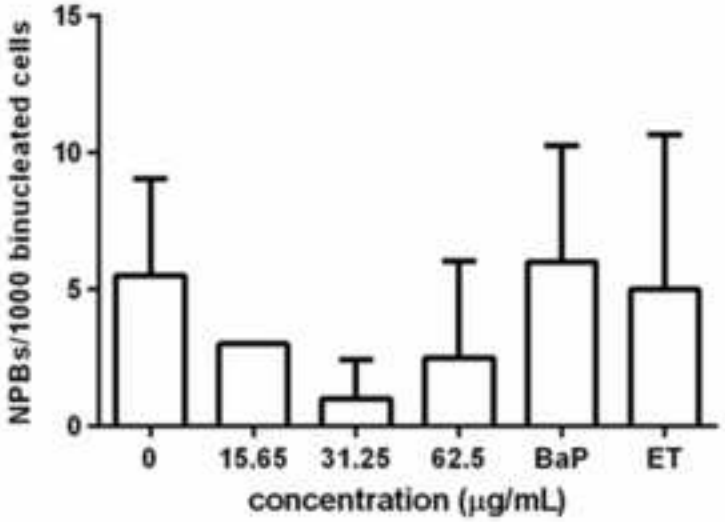


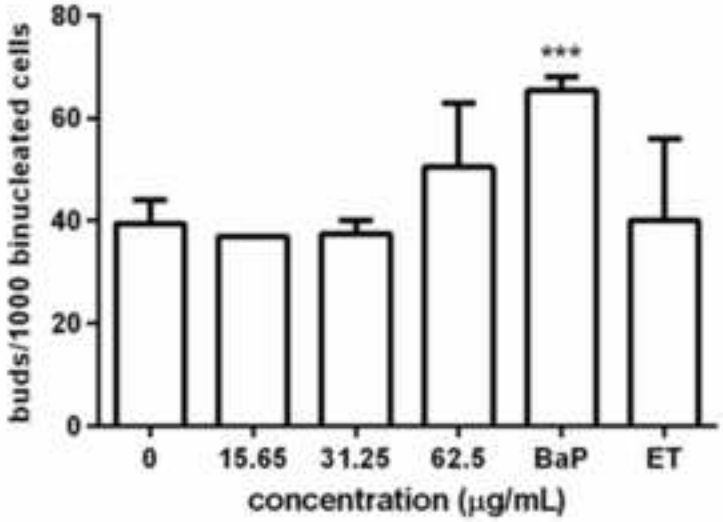
Figure

Figure 2



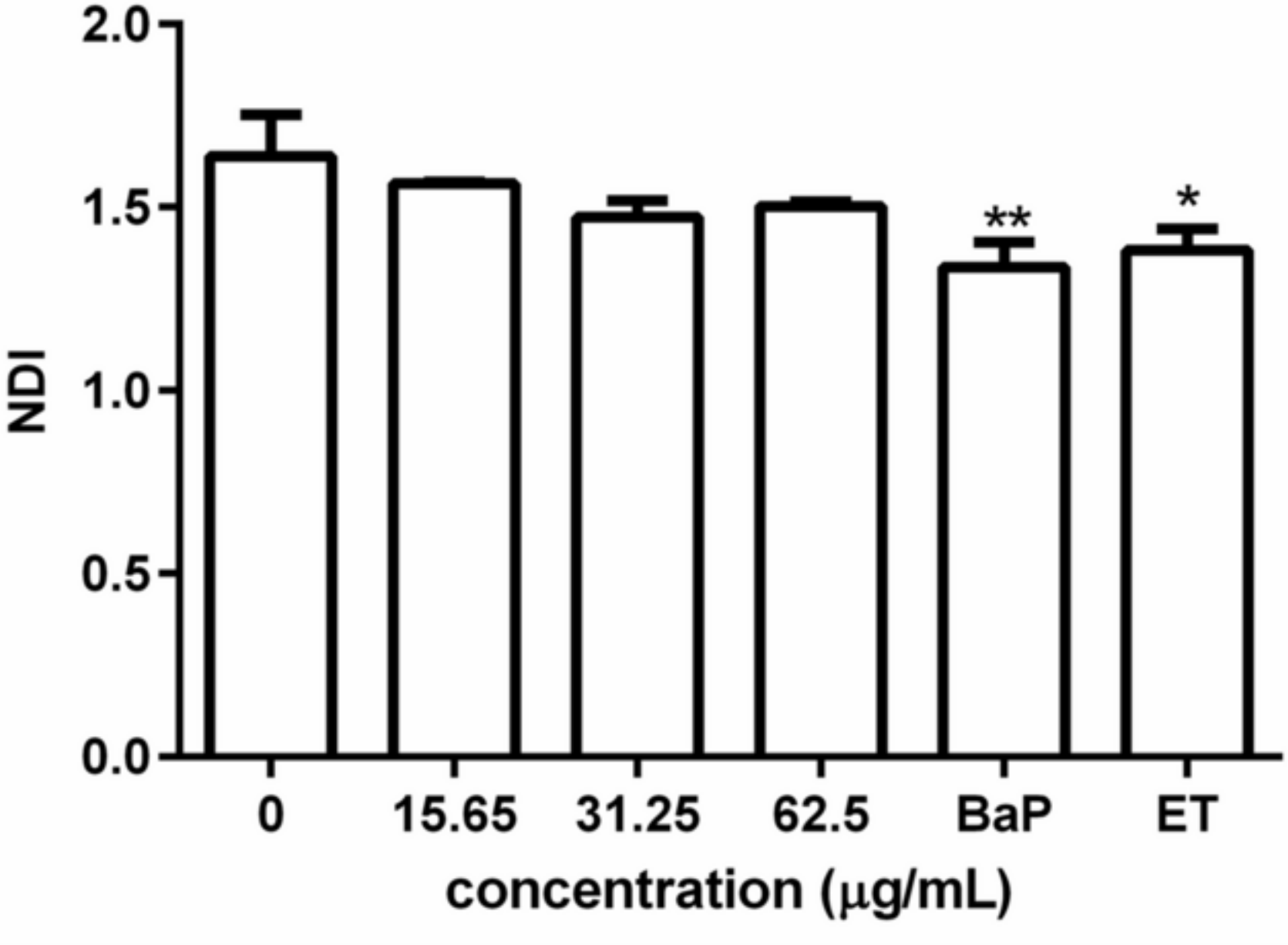






Figure

Figure 3



CAPÍTULO 4 / CHAPTER 4

Sara Maisanaba, Klara Hercog, Susana Aucejo, Ángeles Jos, Bojana Zegura

INDUCTION OF MICRONUCLEI AND ALTERATION OF GENE EXPRESSION BY AN ORGANOMODIFIED CLAY IN HEPG2 CELLS

Enviado a Archives of Toxicology/ Sent to Archives of Toxicology

Archives of Toxicology

Induction of micronuclei and alteration of gene expression by an organomodified clay in HepG2 cells

--Manuscript Draft--

Manuscript Number:	
Full Title:	Induction of micronuclei and alteration of gene expression by an organomodified clay in HepG2 cells
Article Type:	Original Article
Corresponding Author:	Sara Maisanaba SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Sara Maisanaba
First Author Secondary Information:	
Order of Authors:	Sara Maisanaba
	Klara Hercog
	Natalia Ortuño
	Angeles Jos
	Bojana Zegura
Order of Authors Secondary Information:	
Funding Information:	Agencia de Innovación y Desarrollo de Andalucía (ES) (AGR5969) — Mrs Sara Maisanaba
	Javna Agencija za Raziskovalno Dejavnost RS (SI) (P1-0245) — Not applicable
Abstract:	Clay2 is an organomodified montmorillonite developed by the Technological Institute of Packaging, Transport and Logistic (ITENE) in order to improve polymeric materials used in food packaging. There is not much known on Clay2 toxic potential, particularly at DNA level, therefore it is mandatory to assess its toxicity prior to its commercialization. In the present study the human hepatoma cell line (HepG2) was exposed to non-cytotoxic concentrations of Clay2 and the genomic instability was studied with the Cytokinesis block micronucleus cytome assay, by determining the formation of micronuclei (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). Moreover, the expression of various genes involved in the mechanisms of its action using the real-time quantitative PCR was studied. The results obtained provide the evidence that Clay2 is potentially genotoxic as it increased the frequency of micronuclei. In addition it deregulated genes involved in the metabolism, immediate-early response/signaling, DNA damage and oxidative stress showing new valuable information on the cellular response to Clay2. Nonetheless, further studies are highly needed to elucidate the molecular mechanisms of clays toxicity.
Suggested Reviewers:	Alja alja.stern@gmail.com Stern alja.stern@gmail.com Has expertise in genetic toxicology
	Goran Gajski ggajski@imi.hr Has expertise in genetic toxicology and human exposure to different substances like

	pharmaceuticals, toxins, etc.
	Maria Jose Ruiz M.Jose.Ruiz@uv.es She has expertise in toxicology

Induction of micronuclei and alteration of gene expression by an organomodified clay in HepG2 cells

Sara Maisanaba[1*], Klara Hercog[2], Natalia Ortuño[3], Ángeles Jos[1], Bojana Zegura[2]

[1] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[2] National Institute of Biology, Department for Genetic Toxicology and Cancer Biology, Vecna pot 111, 1000 Ljubljana, Slovenia.

[3] Area of Packaging Materials and Systems, ITENE, C/Albert Einstein 1, 46980 Paterna, Valencia, Spain.

*Corresponding author:

Sara Maisanaba

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: saramh@us.es

Tel: +34 954 556762

Abstract

Clay2 is an organomodified montmorillonite developed by the Technological Institute of Packaging, Transport and Logistic (ITENE) in order to improve polymeric materials used in food packaging. There is not much known on Clay2 toxic potential, particularly at DNA level, therefore it is mandatory to assess its toxicity prior to its commercialization. In the present study the human hepatoma cell line (HepG2) was exposed to non-cytotoxic concentrations of Clay2 and the genomic instability was studied with the Cytokinesis block micronucleus cytome assay, by determining the formation of micronuclei (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). Moreover, the expression of various genes involved in the mechanisms of its action using the real-time quantitative PCR was studied. The results obtained provide the evidence that Clay2 is potentially genotoxic as it increased the frequency of micronuclei. In addition it deregulated genes involved in the metabolism, immediate-early response/signaling, DNA damage and oxidative stress showing new valuable information on the cellular response to Clay2. Nonetheless, further studies are highly needed to elucidate the molecular mechanisms of clays toxicity.

Keywords: modified clay; cytokinesis block micronucleus assay; real-time quantitative PCR; HepG2

Introduction

Nearly all of the food and drink that we buy and consume is packed in some way. The main function of food packaging is to protect and preserve the food, to maintain its quality and safety, and to reduce food waste (Bradley et al. 2011). Currently, in order to create new food packaging materials to enhance the perdurability of the food, the incorporation of natural materials into the polymeric matrix, as clays, is presented as a great alternative. The final result is a new product known as nanocomposite, which comes from the disposition of the clay platelets in the polymer (Arora and Padua 2010).

Montmorillonite (Mt) is a layered mineral clay widely used in various science fields due to its good swelling capacity, high specific surface area, good cation-exchange capacity, high platelet aspect ratio, strong adsorptive power, high structural stability, chemical inertia, strong capacity to form stable suspensions and ease with which its surface can be modified (Baek et al. 2012; Mallakpour and Dinari 2012). For these reasons, it is one of the most promising options used in food packaging to obtain improved materials.

However, a simple dispersion of pure Mt, commercially known as Cloisite ®Na^+ (CNa^+), in a polymer matrix will not produce a nanocomposite with better properties compared to those of the bulk material due to the poor interfacial interactions between the hydrophilic reaction sites of clays and the highly hydrophobic polymer chains (Pisticelli et al. 2010), being necessary a chemical change in the clay structure, yielding to organo-modified clays. Cation exchange is one of the most useful chemical modification procedures used to prepare more organophilic clays (Pavlidou and Papaspyrides 2008), and quaternary ammonium salts are one of the most used options. Several quaternary ammonium salt-organomodified clays are available on the market, and many more are under development, such as Clay2, a modified Mt with acetylcholine (ACO) chloride and hexadecyltrimethyl-ammonium bromide (HDTA) (Jordá-Beneyto et al. 2014) that was developed by the Technological Institute of Packaging, Transport and Logistic (ITENE).

Due to the potential presence of organomodified clays such as Clay2 in food packaging materials their thorough toxicological assessment is required in order to determine the absence of toxicity for the consumers. In the scientific literature there is scarce information on the *in vitro* toxic profile of Clay2 tested in different cell lines, and no *in vivo* data. Cytotoxic effects were determined by different endpoints like neutral red uptake, MTS reduction assay and protein content in human cell lines such as HepG2 (hepatic), Caco-2 (intestinal) and HUVEC (endothelial), demonstrating a decrease in the cellular viability after 24 and 48h of exposure in a concentration range 0-125 µg/mL (Houtman et al. 2014; Jordá-Beneyto et al. 2014; Maisanaba et al. 2015a). Moreover, Clay2 induced DNA damage in HepG2 and Caco-2 cells determined by the Comet assay; however, no mutagenic response was obtained with the Ames assay (Houtman et al. 2014; Maisanaba et al. 2015a). Clay2 did not generate reactive oxygen species in HepG2 and Caco-2 cells, however a decrease in GSH content was determined in HepG2 cells exposed to Clay2 (Houtman et al. 2014).

The cytotoxic effects of other organomodified clays, commercially available or under development and the underlying mechanisms of their toxicity have recently been reviewed by Maisanaba et al (2015b).

Several modified clays such as Cloisite®20A and Clay1 showed almost complete absence of toxicity in HepG2 or Caco-2 cells (Houtman et al. 2014, Jordá-Beneyto et al. 2014), while on the contrary a commercially available organomodified clay Cloisite®30B induced cytotoxic, genotoxic and mutagenic effects as well as oxidative stress *in vitro* (Sharma et al, 2010; Maisanaba el al. 2013, 2014, 2015a). These different responses support the idea that a case by case toxicological evaluation is needed. Currently, a genotoxicity evaluation is required by some regulatory and advisory bodies for authorization purposes (EFSA 2011; Kirkland et al. 2014) by means of a core set of tests. Among these assays, the micronucleus test is one of the main genotoxicity tests proposed, but for modified clay minerals, in general, the information about it remains very limited.

Due to the scarce information on genotoxic potential of modified clays, the aim of our study was to elucidate whether Clay2 can influence genomic instability of human hepatoma (HepG2) cells by determining the formation of micronuclei and/or other nuclear anomalies, such as buds or bridges with the cytokinesis block micronucleus assay (CBMN). Moreover, the alterations in the expression patterns of a wide range of selected genes involved in the main activation/detoxification pathways and mechanisms of toxicity by Clay2 were investigated by real-time quantitative PCR (qRT-PCR).

Materials and Methods

Supplies and chemicals

Minimum essential medium (MEM), TRIzol® reagent, B27 supplement were from Gibco BRL (Paisley, Scotland); fetal bovine serum (FBS), non-essential amino acids (NEAA), penicillin/streptomycin, L-glutamine, phosphate buffered saline (PBS) from PAA (Pasching, Austria). Cytochalasin B (Cyt-B), acridine orange (AO), dimethyl sulfoxide (DMSO [CAS 67-68-5]), trypsin, etoposide (ET) [CAS 33419-42-0] and benzo(*a*)pyrene (B(*a*)P) [CAS 50-32-8], were obtained from Sigma–Aldrich (St. Louis, USA). High Capacity cDNA Archive Kit and Taqman Gene Expression Assays were from Applied Biosystems (Forest City, CA, USA), TaqMan Universal PCR Master Mix from Applied Biosystems (Branchburg, NJ, USA), and Human GAPDH from Applied Biosystems (Warrington, UK).

Modified clay and characterization

Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) (C19H42BrN, 364.46 g/mol, 98%) was supplied from Cymit Quimica S.L. (Spain), acetylcholine chloride (ACO) (C7H16ClNO2, 181.66 g/mol, P99%) was provided by Sigma- Aldrich (Spain).

The non commercial organomodified clay, Clay2 (Fig.1), has been developed and characterized by ITENE as described previously (Jordá-Beneyto et al. 2014). Briefly, the organomodified clay was prepared by a cation-exchange method, which consists on a displacement of the sodium cations of CNa^{+} with the ammonium cations of the above mentioned salts, obtaining Clay2.

The organomodified clay was characterized by thermogravimetric analysis (TGA), Fourier Transform InfraRed (FTIR) and X-ray diffraction (XRD) as described in Jordá-Beneyto et al (2014).

Clay test solutions

The test concentrations for the clay were selected according to previous dispersion experiments in order to avoid interferences with the measurement system as described Maisanaba et al. (2013). Test solutions were prepared in serum-free medium supplemented with B27. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

Cell culture

HepG2 (human hepatocellular carcinoma epithelial cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% of FBS (Gibco, New Zealand), 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin/ streptomycin (Gibco, New Zealand). Cells were grown at 37°C and 5% CO_2 in humidified atmosphere. Cells were used at passages between 12 and 18.

Cytotoxicity assay

HepG2 cells were seeded onto 96-well tissue-culture plates at a density of 4000 cells/well, and incubated at 37°C for 24h prior to exposure. From the initial stock solutions, serial dilutions of Clay2 in the medium without FBS supplemented with 2% B27 were prepared. Culture medium without clay was used as control group. Before testing genotoxic potential of Clay2 the viability of HepG2 cells was determined to avoid false positive results due to cytotoxicity and not genotoxicity. Growth medium was replaced with freshly prepared medium containing serial dilutions of Clay2 (1.56, 15.6 and 31.25 µg/mL) and incubated at 37 °C for 4 and 24h. Cytotoxicity of Clay2 was determined with the tetrazolium salt reduction (MTS) (CellTiter96 AQueous Non Radioactive Cell Proliferation Assay; Promega, Madison, USA) according to the manufacturer's instructions. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt) tetrazolium compound added to the medium is reduced by cells into a colored formazan product soluble in culture medium, which is directly measured with the spectrophotometer at 490 nm after 3h of incubation in the dark. Cell viability was determined by comparing the optical density of the wells containing the cells treated with the Clay2 with those of the control group. Two independent experiments were performed, each in five replicates per treatment point.

Cytokinesis block micronucleus assay (CBMN)

Prior to the treatment, the cells were seeded onto 6-well tissue culture treated plates (400 000 cells/ well) and incubated for 24h to attach. The medium was then replaced with fresh complete medium

containing non-cytotoxic concentrations of Clay2 (3.9, 7.8 and 15.6 µg/mL) and incubated for 24h. B(*a*)P (30 µM) and ET (1 µg/mL) were used as positive controls.

The CBMN assay was performed following the steps described in Straser et al. (2011). Briefly, after the treatment, the cells were washed twice with 1x PBS and fresh medium with Cyt-B (3 µg/ml) was added for 26h at 37°C. Subsequently, the cells were washed, trypsinized, incubated in a cold hypotonic solution (75 mM KCl) for 5 min and fixed with a mix of methanol/acetic (3:1; v/v) and formaldehyde 37%. The fixed cells were put on microscope slides and air dried, stained with AO (10 µg/ml) and examined under the fluorescence microscope (Olympus BX61, Japan). Micronuclei (MN), nucleoplasmic bridges (NPBs) and cells with nuclear buds (NBUDs) were counted in 1000 binucleated cells (BNC) per experiment and were scored according to the criteria published by Fenech et al. (2000), in two independent experiments. In addition, the frequency of the binucleated cells with MN (MNed cells), was counted. The nuclear division index (NDI) was determined by scoring 1000 cells with one to four nuclei and was calculated using the formula [M1+ 2M2+3 (M3 + M4)]/500, where M1–M4 represent the number of cells with one to four nuclei, respectively, and M3 and M4 are equally considered to be in their third cycle (Fenech 2000).

Real-time quantitative PCR (qRT-PCR) analysis

After the incubation, the cells were washed with 1x PBS and total RNA was isolated using TRIzol® reagent, according to the manufacturer's protocol with minor modifications described in Maisanaba et al (2015c). Briefly, glycogen (2 mg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at -20°C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water. The RNA was transcribed to cDNA using 1 µg of total RNA and cDNA High Capacity Kit, according to the manufacturer's protocol. Gene expression was quantified with real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA) using TaqMan Universal PCR Master Mix and the Taqman Gene Expression Assays (all from Applied Biosystems). The following Taqman Gene Expression Assays were used: *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1), Hs01054797_g1; *CYP1A2* (cytochrome P450, family 1, subfamily A, polypeptide 2), Hs00167927_m1; *CYP3A4* (cytochrome P450, family 3, subfamily A, polypeptide 4) Hs00604506_m1; *GSTA1* (glutathione S-transferase alpha 1), Hs00275575_m1; *UGT1A1* (UDP glucuronosyltransferase 1 family, polypeptide A1), Hs02511055_s1; *FOS* (FBJ murine osteosarcoma viral oncogene homolog), Hs04194186_s1; *JUNB* (jun B proto-oncogene) Hs00357891_s1; *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) Hs00153408_m1; *TGFB2* (transforming growth factor, beta 2) Hs00234244_m1; *CDKN1A* (cyclin-dependent kinase inhibitor 1A') Hs00355782_m1; *CHEK1* (checkpoint kinase 1) Hs00967506_m1; *ERCC4* (excision repair cross-complementing rodent repair deficiency, complementation group 4) Hs00193342_m1; *GADD45A* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; *MDM2* (Mdm2, 'MDM2 oncogene, E3 ubiquitin protein ligase), Hs01066930_m1; TP53 (tumor protein P53), Hs01034249_m1; *BAX* (BCL2-associated X protein), Hs00180269_m1; *BCL2* (B-cell CLL/lymphoma 2), Hs00608023_m1; *CASP3* (caspase 3, apoptosis-related cysteine peptidase), Hs00234387_m1; *CASP8* (caspase 8, apoptosis-related cysteine peptidase), Hs01018151_m1; *CASP9* (caspase 9, apoptosis-related cysteine peptidase), Hs00609647_m1;

FAS (Fas cell surface death receptor), Hs00236330_m1; *CAT* (catalase), Hs00156308_m1; *GCLC* (glutamate-cysteine ligase, catalytic subunit), Hs00155249_m1; *GPX1* (glutathione peroxidase 1), Hs00829989_gH; *GSR* (glutathione reductase), Hs00167317_m1; *SOD1A* (superoxide dismutase 1), Hs00533490_m1. Amplification of *GAPDH* probe (Human Endogenous Controls, Cat. No.: 4310884E, Applied Biosystems, USA) was performed as an internal control. The conditions for the PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using an accurate quantification taking into account a control pattern (10x, 100x, 1000x, 10000x) and a normalization with the control group. The expression levels of target mRNAs were normalized to the *GAPDH* mRNA level. Two independent experiments were performed each time in three parallels. An up-regulation and down-regulation of ≥ 1.5 and ≤ 0.7-fold change, respectively, compared to control was considered a positive response.

Statistical analysis

The statistical analyses were performed with GraphPad Prism 5 software. Dunnett's test was used for multiple comparisons versus the control in the MTS assay. In the CBMN assay Chi-square test was used to compare the number of MNs, NBUDs, and NPBs between the control group and Clay2 exposed cells. When calculating differences between samples for the gene expression profiles, statistical significance between treated group and control was determined by Two-tailed Student's t-test comparison. $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant.

Results

Viability of HepG2 exposed to Clay2

After 4h exposure of HepG2 cells to Clay2 no influence on cell viability was observed, while after 24h statistically significant decrease of cell viability was determined at 31.25 µg/mL (Fig. 2); therefore, the non-cytotoxic concentrations up to 15.6 µg/mL were used in further experiments.

Genotoxicity of Clay2 in HepG2: Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of Clay2 in HepG2 cells was further evaluated using the CBMN assay (Fig. 3). Following 24 h exposure, Clay 2 induced statistically significant increase in the frequency of MN (2.7-fold increase) in BNCs, as well as in MNed cells (approximately 2.5-fold higher compared to control group) at the highest concentration assayed (15.6 µg/mL). Moreover, slight though statistically significant changes were obtained for BUDs (1.7-fold increase) at 15.6 µg/mL, while no changes in the frequency of NPBs compared to non-treated control were determined.

A positive control B(*a*)P (2.5 µM) induced statistically significant increase in frequencies of MNed cells (2.5-fold), MN (2.6-fold), NBUDs (1.3-fold), but not NPBs, while a positive control ET (1µg/mL) statistically significantly induced the number of MNed cells (3.7-fold) and MN (4.2-fold) (Fig.4). NDI was slightly decreased in cells exposed to Clay2 and was statistically significant at 7.8 µg/mL. Both positive controls, B(*a*)P and ET, decreased NDI (Fig.5).

Effect of Clay2 on mRNA expression

The mRNa expression of a wide range of selected genes was analyzed after 4 and 24h of exposure to 1.56 and 15.6 µg/mL of Clay2 by quantitative real-time PCR (Table 1). The values presented in the Table 1 represent relative change in the gene expression of the selected genes compared to control group (untreated cells).

Effect of Clay2 on mRNA expression of genes involved in metabolism

In HepG2 cells exposed to Clay2 for 4h, the mRNA levels of the genes studied did not show any change compared to the control group. However, after 24h of exposure all studied genes from the CYP450 family, *CYP1A1* (5.28±0.68), *CYP1A2* (6.41±0.16) and *CYP3A4* (4.33±3.07) were significantly up-regulated at 15.6 µg/mL of Clay2, *CYP1A2* was more than 1.7-fold up-regulated even at 1.56 µg/mL. The expression of gene *GST1A* from the detoxification pathway was 1.5-fold increased at 15.6 µg/mL of Clay2 compared to control, while the expression of *UGT1A1* was not statistically significantly changed at applied concentrations.

Effect of Clay2 on mRNA expression of immediate –early response/signaling genes

After the exposure of cells to Clay2 for 4h, only the expressions of *FOS* and *JUNB* were significantly down-regulation at both concentrations tested. On the contrary these two genes, *FOS* (5.08±0.57) and *JUNB* (1.74±0.34) were statistically up-regulated after 24h at the higher tested concentration. Also the expressions of *MYC* and *TGFB2* were increased (approximately 2-fold) compared to control at 15.6 and 1.56 µg/mL, respectively.

Effect of Clay2 on mRNA expression of DNA damage responsive genes

Clay2 did not deregulate the mRNA levels of the genes involved in the response to DNA damage (*TP53, MDM2, CDKN1A, GADD45A, CHEK1* and *ERCC4*) after 4h of exposure, while after 24h it up-regulated (≥ 1.5-fold) the expression of *TP53* and its downstream regulated genes *MDM2*, *CDKN1A*, *GADD45A* at 15.6 µg/mL. The mRNA level of *CHECK1* was decreased compare to control (0.68±0.26). Clay2 did not affect the expression of *ERCC4* at applied tested conditions.

Effect of Clay2 on mRNA expression of oxidative stress responsive genes

In hepatoma cells exposed to Clay2 for 4h, the mRNA levels of the oxidative stress responsive genes studied were not changed compared to control group. After 24h, only *CAT* presented an important down-regulation at both concentrations used, and *GCLC* showed a significant up-regulation (2.14-fold change) at the highest one. Clay2 did not affect the expression of other studied genes at applied conditions.

Effect of Clay2 on mRNA expression of apoptosis responsive genes

No changes in the expressions of selected apoptosis/survival responsive genes (*BAX, BCL2, CASP3, CASP8, CASP9* and *FAS*) were observed in HepG2 cells exposed to Clay2 for 4 and 24h under the conditions tested.

Discussion

Clay2 is a novel organoclay developed by ITENE to improve polymer properties used in food packaging. The toxicological data available on this clay are scarce but are required in order to comply with authorization processes. Previous experiments have shown inconclusive, contradictory and equivocal results on Clay2 genotoxic activity *in vitro*. Clay2 was not mutagenic in *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA104 (Maisanaba et al. 2015a); however, it induced DNA strand breaks in Caco-2 and HepG2 cell lines (Houtman et al. 2014). In such cases, the European Food Safety Authority states that it is appropriate to conduct further testing *in vitro* (EFSA 2011). The CBMN assay provides an additional strategy with a different genetic endpoint. Its purpose is to identify substances that cause structural and numerical chromosomal damage in cells that have undergone cell division during or after the exposure to the test substance (EFSA 2011). In the present study we have shown that Clay2 has altered the frequency of MNed cells and MN. Taken together all these data suggest that Clay2 could be considered genotoxic *in vitro*. However, based on these results it is not possible to conclude whether the modifiers introduced to the molecular formula of the raw clay are responsible for the genotoxicity observed especially as the raw clay, CNa^+, has also increased the frequency of MN in the same experimental model but at higher concentrations (Maisanaba et al. 2015c).

In addition to the classical toxicology assays we used transcriptomic analyses to identify molecular pathways potentially involved in Clay2 toxicity. To our knowledge no similar studies were conducted so far on modified clays; therefore we determined the influence of Clay2 on the expression of a wide range of selected genes after short (4h) and long (24h) exposure.

Cytochrome P450 enzymes are responsible for the metabolism of a wide range of endogenous compounds, as well as xenobiotics including drugs, environmental pollutants, and dietary products (Nebert and Russell 2002). CYP1A1 and CYP1A2 enzymes are not only involved in drug metabolism but are also associated with bioactivation of many pro-carcinogens (Ma and Lu 2007). CYP3A4 is the most abundantly expressed P450 enzyme in the human liver and is involved in the metabolism of approximately half of all currently used prescription drugs (Thummel and Wilkinson 1998; Hao et al. 2011). Therefore, it is important to know the alterations in the expression of CYP enzymes at the gene level. Clay2 induced dose and time dependent up-regulation of all three studied *CYP* genes. Similar expression pattern has already been observed with the raw clay, CNa^+ (Maisanaba et al. 2015c). Clay2 deregulated also the expression of *GST1A1*, which belongs to the phase II enzymes (mainly related to the metabolic detoxification processes), while the expression of *UGT1A1* was slightly down-regulated but

was not significant. Previously, Maisanaba et al. (2015c) reported that also CNa^+ enhanced *GST1A1* expression and significantly decreased *UGT1A1* level. Altogether these results clearly show that clays can deregulate the expression of genes involved in the metabolism, which is especially important if we consider the fact that in real exposure interactions with other xenobiotics can occur. Consequently the activation/detoxification of xenobiotics can be changed.

Clay2 deregulated the expression of *FOS*, *JUNB* and *MYC*, immediate-early response/signaling genes by down-regulating *FOS* and *JUNB* after short time exposure and by up-regulating all three studied genes after longer exposure. *FOS* and *JUNB* are components of the activator protein 1 (AP-1), which consists of Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (JunB, c-Jun, and JunD), and activating transcription factor (ATF) families (Yang et al. 2003). The induction of *FOS* is known to be indicative for DNA-damage and is increased by a wide variety of DNA-damaging agents (Hollander and Fornace 1989). *JUNB* gene is an immediate-early or primary response gene that is induced by polypeptide growth factors, cytokines, and chemical agents in various cell types, including liver (Hsu et al. 1992). *MYC* genes are also key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumors (Adhikary and Eilers 2005). The results obtained in the present study are consistent with those obtained with CNa^+ (Maisanaba et al. 2015c). TGFB2, a potent anti-inflammatory cytokine, belongs to the transforming growth factor Bs (TGF-Bs) that regulate a wide variety of cellular processes, and were shown to inhibit epithelial cell proliferation by delaying or arresting progression through the late portion of G1 (Roberts and Sporn 1993). Clay2 up-regulated the expression of *TGFB2* after 24h, similarly as CNa^+ (Maisanaba et al. 2015c). Thus, as many other xenobiotics, clays can also affect cell signaling pathways with consequences on the cell faith.

Most of the DNA damage responsive genes studied (*TP53*, *CDKN1A*, *GADD45A* and *MDM2*) were up-regulated at the highest concentration of Clay2, while CNa^+ deregulated only *CDKN1A* and *GADD45A* (Maisanaba et al. 2015c). The tumor-suppressor gene, *TP53*, has several important functions such as activation of DNA repair proteins, cell cycle arrest, differentiation and initiation of apoptosis, depending on the level of DNA damage (Vogelstein et al. 2000). The up-regulated expression of *TP53* and its downstream regulated genes, *CDKN1A* and *GADD45A*, can be associated with the cell cycle arrest due to DNA damage induced by Clay2. MDM2 that enhances the tumorigenic potential of the cells and promotes cell survival and cell cycle progression was also up-regulated at mRNA level by Clay2. On the other hand, *ERCC4* and a Ser/Thr kinase *CHEK1*, that are involved in nucleotide excision repair and mediation of the cellular response to DNA-damage, respectively, did not show any change upon Clay2 exposure.

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses (Betteridge 2000) and can be induced by a wide range of xenobiotics. Among the cellular antioxidant defenses, SOD, CAT and the glutathione (GSH) enzymatic system have a main role. The alterations observed in genes involved in the response to oxidative stress induced by Clay2 followed the same pattern than those induced by CNa^+, namely down-regulation of *CAT*, up-regulation of *GCLC* and no changes in other genes studied (Maisanaba et al. 2015c). CAT

enzyme dismutates hydrogen peroxide into water and molecular oxygen. Low levels of *CAT* together with no effects on *GPX* could correlate with enhanced H_2O_2 levels leading to oxidative stress and therefore potential serious tissue damage through oxidative damage and excessive cell death. Previously, Houtman et al. (2014) reported that Clay2 did not increase the level of ROS in exposed HepG2 cells but it decreased the GSH content. In the present study we observed the up-regulation of *GCLC*, the first rate limiting enzyme of glutathione synthesis, which could be explained as a response to the decreased GSH levels observed in HepG2 after Clay2 exposure (Houtman et al. 2014).

Apoptosis, a programmed cell death, is critically important for the survival of multicellular organisms by getting rid of damaged or infected cells that may interfere with normal function (Vicencio et al. 2008). Two major pathways initiate apoptosis: the intrinsic (or mitochondrial) and the extrinsic pathways. In the present study we measured the expression of several genes involved in the intrinsic/extrinsic apoptotic processes as it is not know whether Clay2 can induce apoptosis. Although the results showed that none of the genes from the apoptotic pathways were deregulated by Clay2, the induction of apoptotic processes cannot be excluded as these data shown only that at exposure conditions Clay2 did not interfere with studied genes from apoptotic pathways at the transcriptional level.

Previously, it has been described that Clay2 decreased cell viability of intestinal Caco-2 cells, hepatic HepG2 cells and endothelial HUVEC cells with EC_{50} values of 55, 88 and 42 µg/mL, respectively, measured with the MTS assay (Houtman et al. 2014; Maisanaba et al. 2015a), but the decrease was not associated to apoptosis. CNa^+ however, induced an up-regulation of *BCL2* suggesting the suppression of apoptosis, while on the other hand it up-regulated *CASP3* indicating potential involvement of intrinsic pathway of apoptosis in CNa^+ toxicity (Maisanaba et al. 2015c). The profile of the effects induced by the different modified clays can vary, thus a case by case toxicological evaluation is required. For example no increases of caspase-3/7 activity in HepG2 cells treated with CNa^+, Cloisite ®93A (Lordan et al. 2010), and Cloisite 30®B (Maisanaba et al. 2013), the raw Mt and two Mt-organomodified clays, respectively, were detected. Janer et al. (2014), however, observed that CNa^+ induced statistically significant increase of caspase-3/7 activity as well as translocation of membrane phospholipid phosphatidyleserine to the plasma membrane indicating the induction of apoptotic processes in HepG2 cells.

Overall, when comparing the effects induced by raw (CNa^+) (Maisanaba et al. 2015c) and modified (Clay2) clay it can be seen that Clay2 was more cytotoxic and had stronger genotoxic potential than CNa^+ as it at lower concentrations induced the formation of micronuclei and alteration of different genes in HepG2 cells. Further research is needed to clarify the molecular mechanism involved in genotoxicity of clays and to identify the risk for humans in potential continuous exposure to low doses of the clays.

Conclusions

The *in vitro* results obtained in the present study indicate that Clay2 is a potential genotoxic compound by the CBMN assay and also induce alterations of a wide range of genes involved in the metabolism, immediate-early response/signaling, DNA damage response and oxidative stress in human hepatoma cells mainly after 24h of exposure. Moreover, at concentrations that induced DNA damage Clay2 did not deregulate genes involved in apoptotic processes, which may result in an additional hazard of Clay2 as cells with damaged DNA are not removed from the population, potentially enhancing the risk of mutations and consequently carcinogenesis. Therefore Clay2 requires a thorough toxicological assessment before its intended used in food packaging.

Acknowledgements

The authors wish to thank Junta de Andalucía (AGR5969) and Slovenian Research Agency (Program P1-0245) for the financial support. Sara Maisanaba also gratefully acknowledges Junta de Andalucía for her predoctoral grant associated to the AGR5969 project.

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Figure captions

Figure 1. Clay2 composition: a) Raw clay structure (Mt) and its modifiers, b) hexadecyltrimethyl ammonium bromide (HDTA) and c) acethylcholine (ACO).

Figure 2. The effect of Clay2 on the viability of HepG2 cells. Viability was determined with the MTS assay after the exposure to different concentrations Clay2 (write here the concentrations) for 4 and 24 h. Significant differences between Clay2 treated cells and the control group is indicated by *P < 0.05, ** P < 0.01(One way ANOVA; Dunnett's test).

Figure 3. Fluorescent micrographs of HepG2 cells exposed to Clay2 (15.6 μg/ml for 24 h) a) binucleated cell, b) binucleated cell with MN, c) binucleated cell with two MNi, d) binucleated cells with NBUD, e) binucleated cells with NBUD and MN and f) binucleated cell with NBP.

Figure 4. Number of binucleated cells with a) micronucleated cells (MNed cells), b) micronuclei (MNi), c) nuclear bridges (NPBs) and d) cells with nuclear buds (NBUDs) per 1000 binucleated HepG2 cells after the exposure to Clay2 (0, 3.9, 7.8 and 15.6 μg/mL) for 24 h. The number of MNed cells shows the number of cells containing MN, while MNi shows the overall number of MN as some cells may contain more than one MN. Significant differences between Clay2 treated cells and the control group (0) is indicated by *P < 0.05, ** P < 0.01, *** P < 0.001, and ****P<0.0001. Benzo(a)pyrene (B(*a*)P; 2.5 μM) and Etoposide (ET; 1 μg/mL) were used as positive controls.

Figure 5. The influence of Clay2 (0, 3.9, 7.8 and 15.6 μg/mL) on the nuclear division index (NDI) of HepG2 cells after 24h exposure. Significant differences between Clay2-treated cells and the control group (0) is indicated by *P < 0.05. Benzo(a)pyrene (B(*a*)P; 2.5 μM) and Etoposide (ET; 1 μg/mL) were used as positive controls.

Table caption

Table 1. Effect of Clay2 on the expression of mRNAs of selected genes involved in the metabolism, immediate-early response/signaling, DNA damage, oxidative stress response and apoptosis/survival in HepG2 cells. The cells were exposed to Clay 2 (Concentrations!) for 4 and 24h. Statistical significance between treated group and control was determined by Two-tailed Student's t-test comparison. $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant; an up-regulation and down-regulation of ≥ 1.5 and ≤ 0.7-fold change, respectively, compared to control was considered a positive response (marked in bold).

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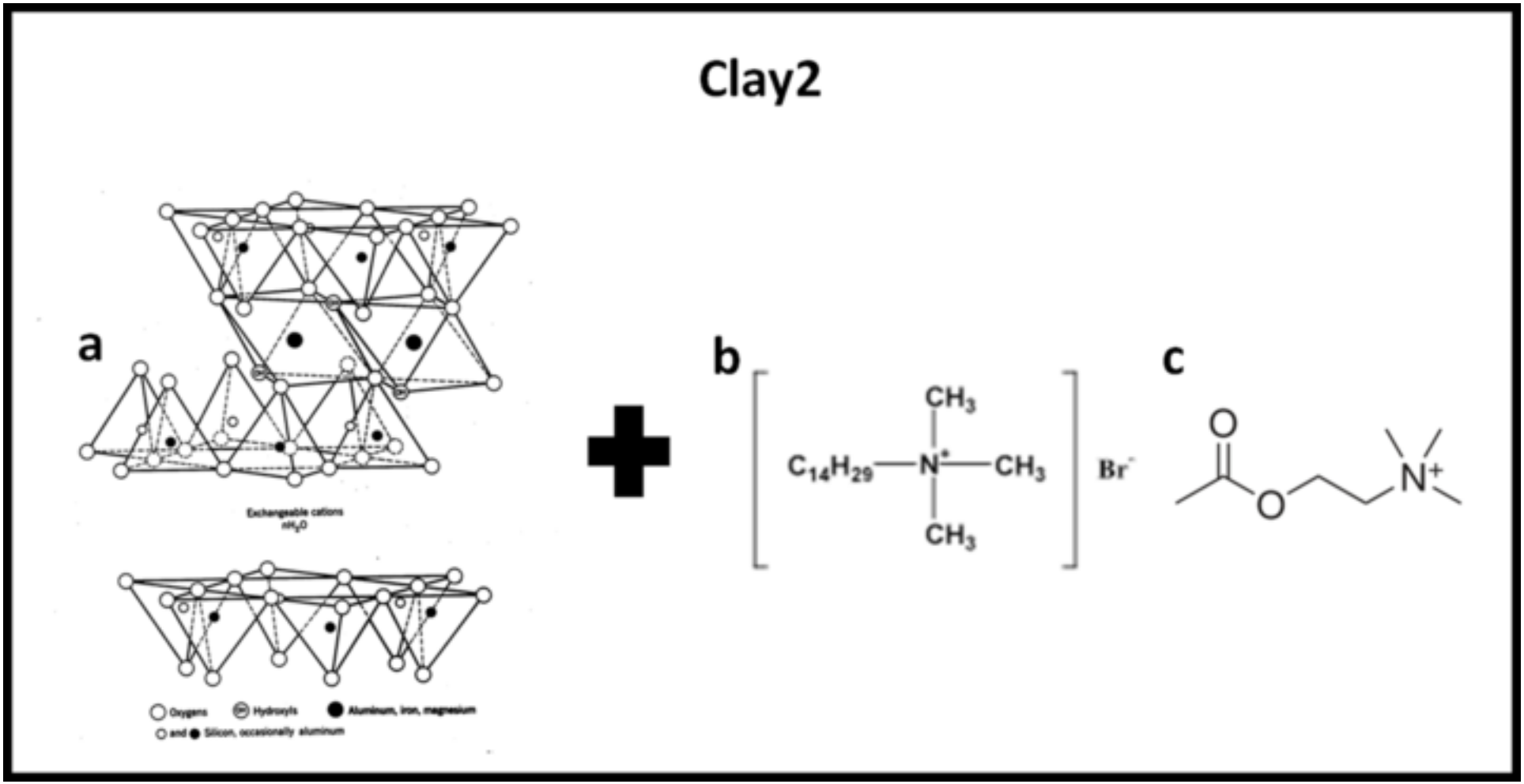

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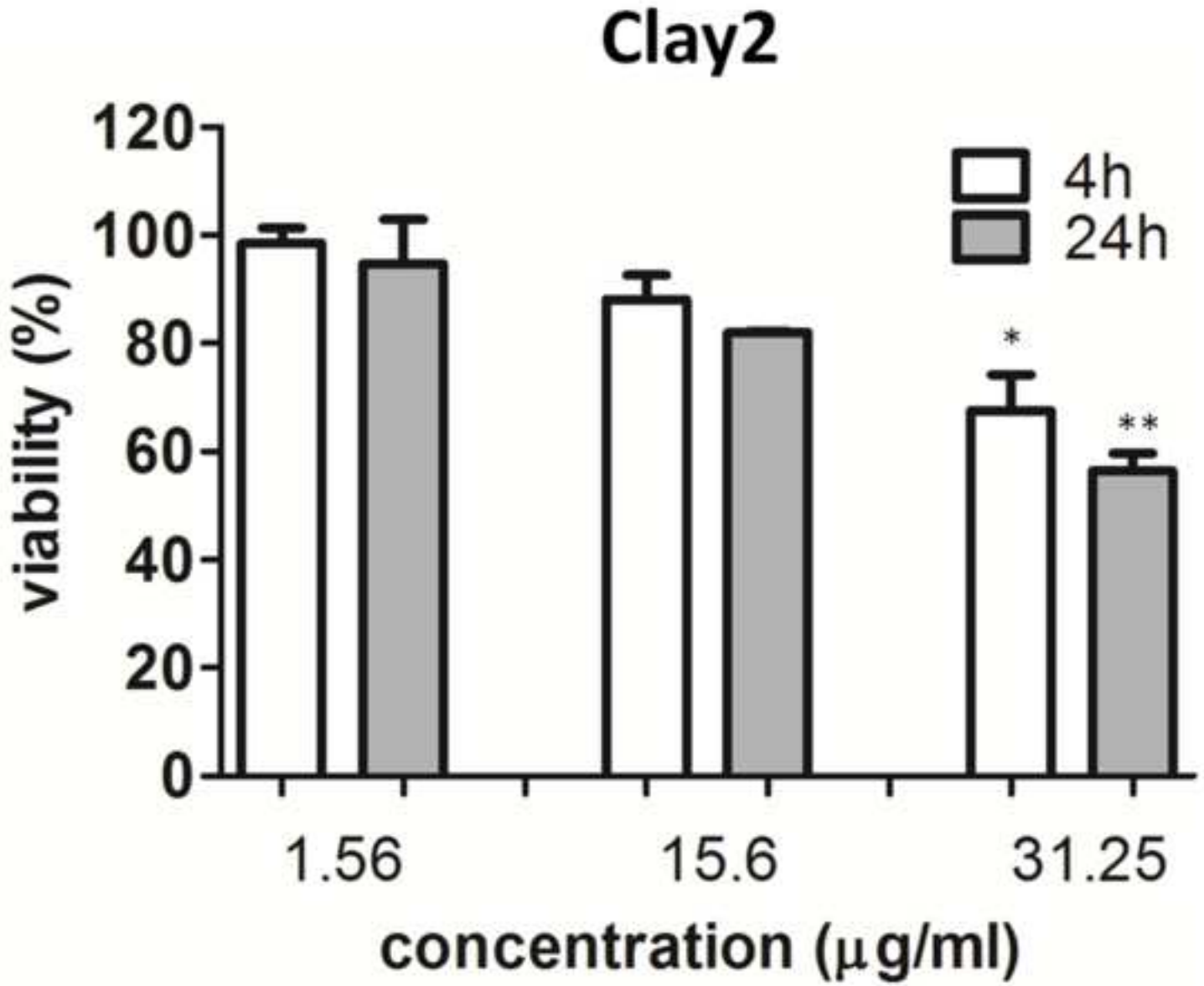

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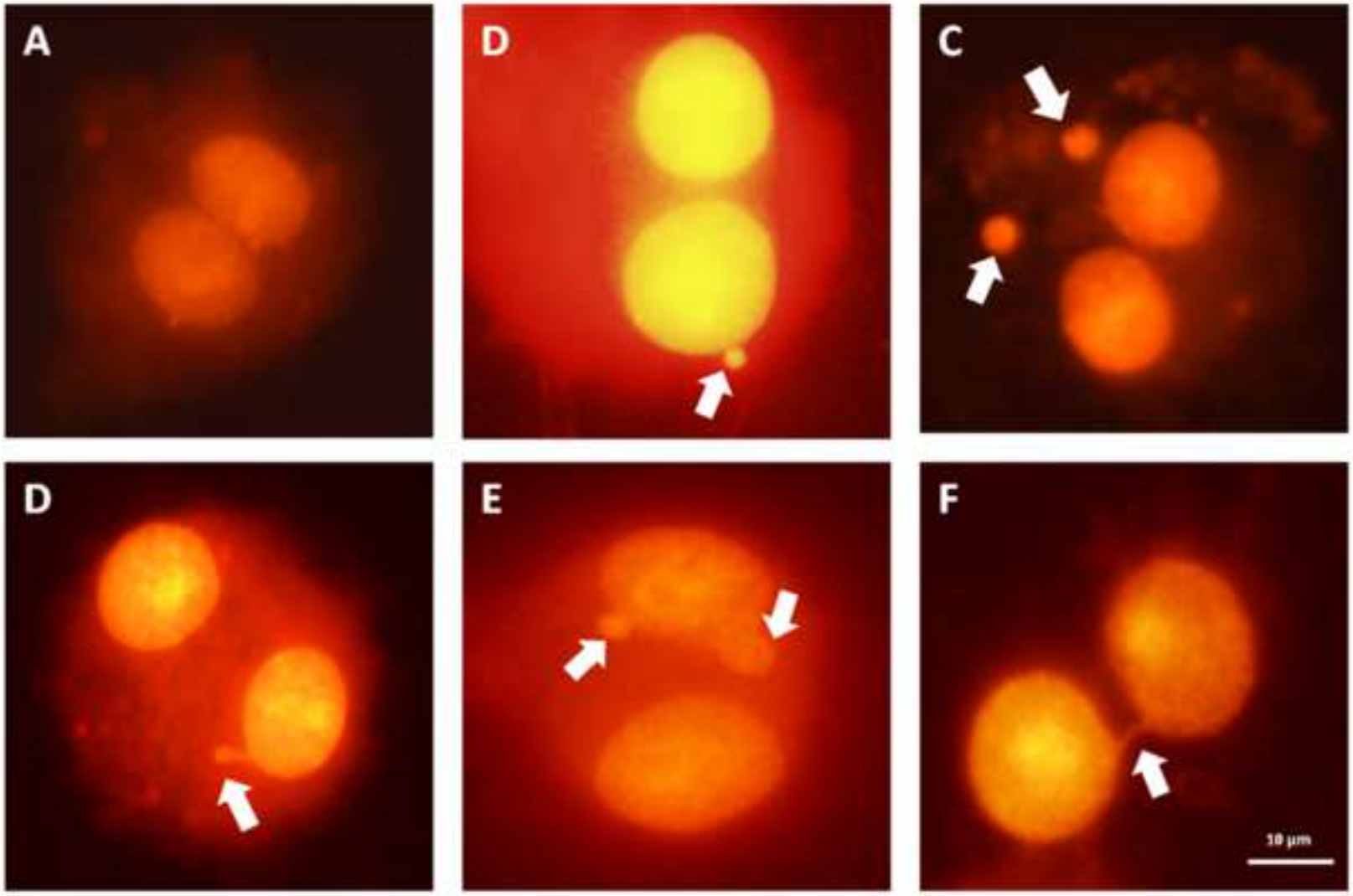

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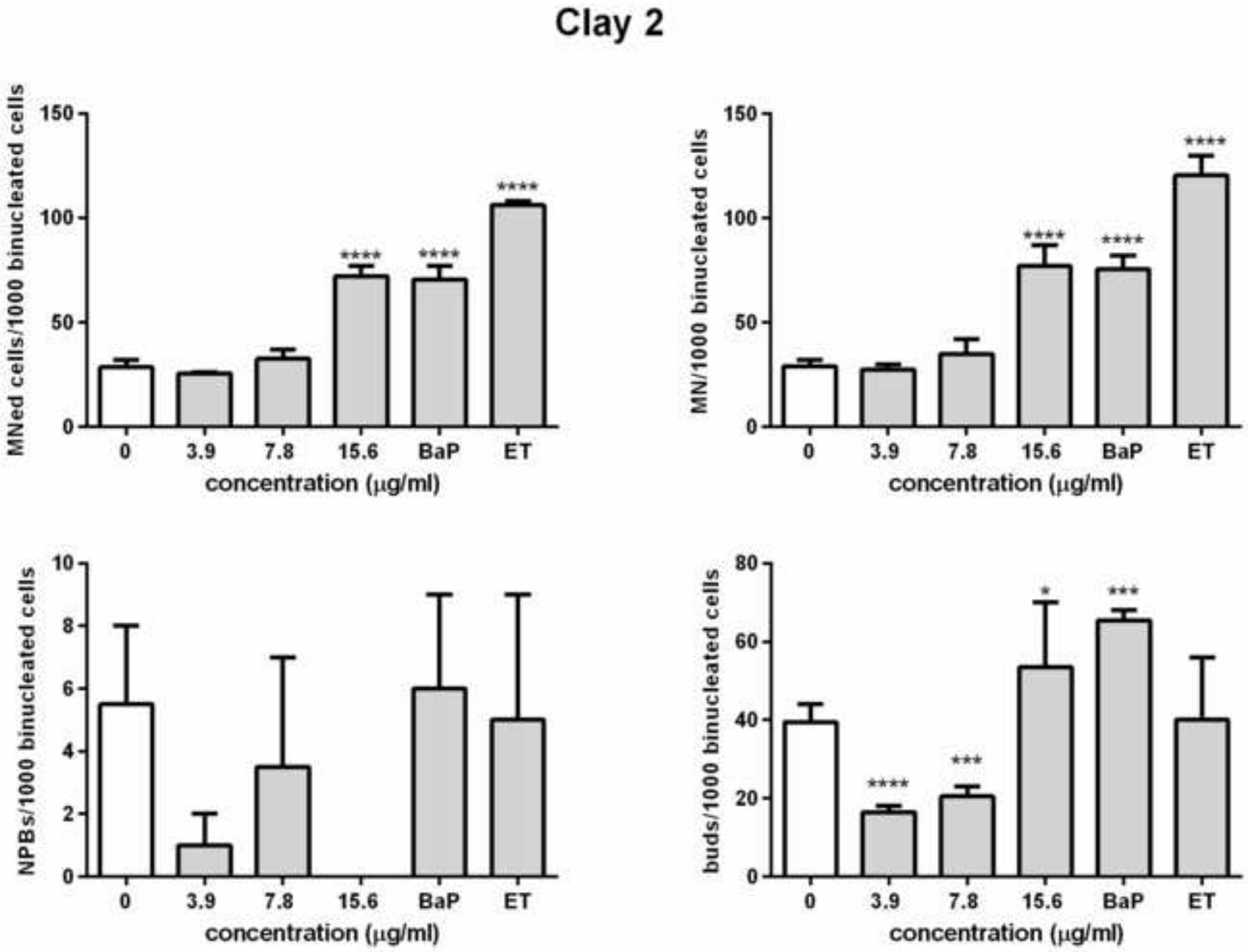

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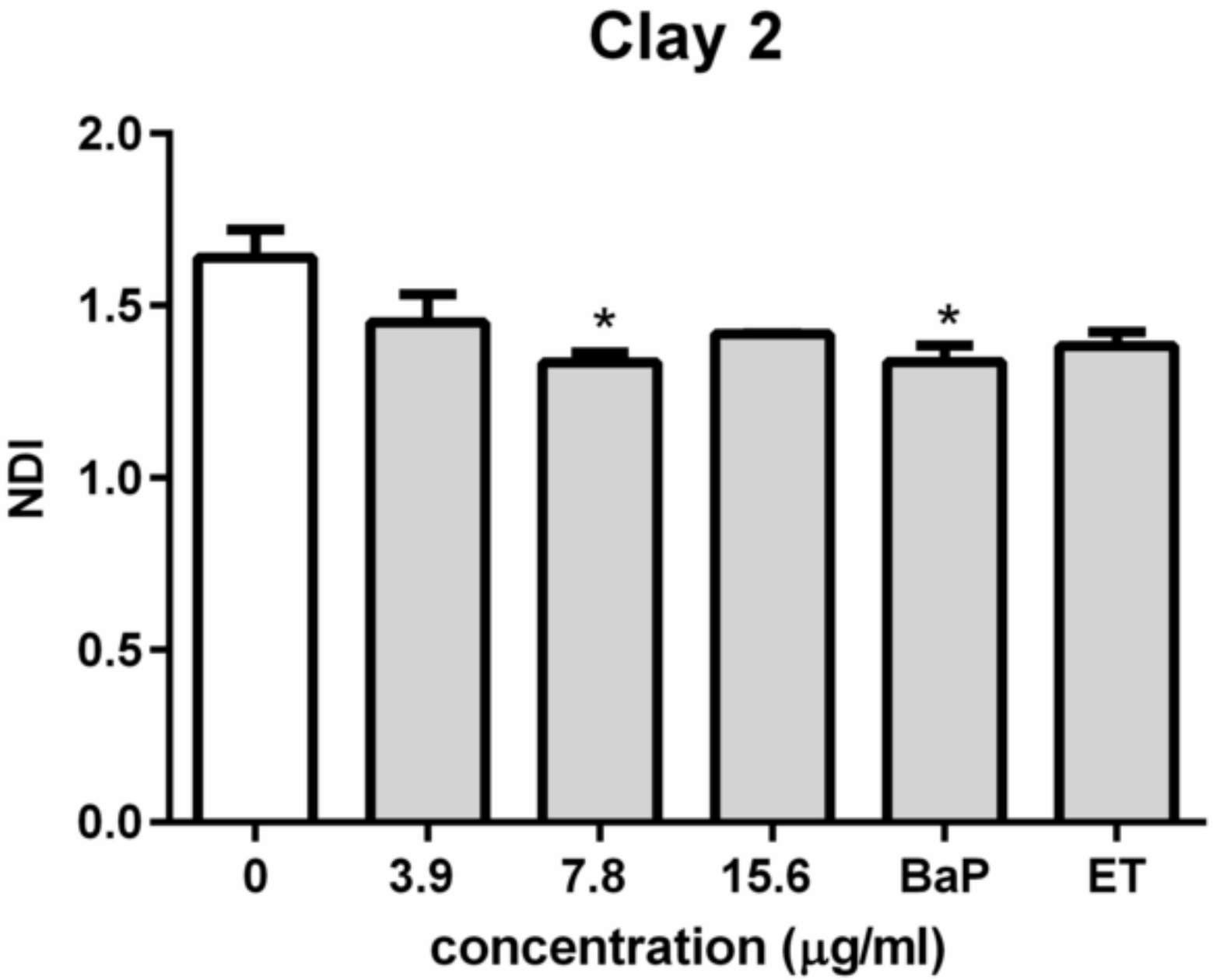

Table

Table 1. Effect of Clay2 on the expression of mRNAs of selected genes involved in the metabolism, immediate-early response/signaling, DNA damage, oxidative stress response and apoptosis/survival in HepG2 cells.

Mechanisms involved	Gene symbol	Clay2 (µg/mL)	4h	24h	Entrez gene name
			Mean±SD	Mean±SD	
Metabolism	***CYP1A1***	1.5	0.89±0.05	1.16±0.22	Cytochrome P450 family 1. Subfamily A. polypeptide 1
		15.6	0.87±0.06	**5.28±0.68****	
		B(a)P 30µM	**1.91±0.43**	**130.32±9.22****	
	CYP1A2	1.5	1.56±0.65	**1.71±0.56**	Cytochrome P450 family 1. Subfamily A. polypeptide 2
		15.6	0.97±0.08	**6.41±0.16*****	
		B(a)P 30µM	**1.76±0.73**	**79.78±15.01****	
	CYP3A4	1.5	1.84±0.04	1.19±0.56	Cytochrome P450 family 3. Subfamily A. polypeptide 4
		15.6	1.13±0.22	**4.33±3.07**	
		B(a)P 30µM	1.11±0.17	**2.66±0.18****	
	GST1A1	1.5	0.76±0.20	1.33±0.32	Glutathione S-transferase alpha 1
		15.6	0.81±0.13	**1.53±0.13***	
		B(a)P 30µM	0.75±0.01***	**0.60±0.25**	
	UGT1A1	1.5	0.58±0.13	0.82±0.27	UDP glucuronosyltransferase 1 family. polypeptide A1
		15.6	0.72±0.13	0.81±0.21	
		B(a)P 30µM	0.97±0.02	**5.57±0.96***	
Inmediate-early response/signaling	***FOS***	1.5	**0.53±0.15***	0.99±0.08	FBJ murine osteosarcoma via oncogene homolog B
		15.6	**0.48±0.12***	**5.08±0.57****	
		B(a)P 30µM	1.23±0.73	**12.49±5.54****	
	JUNB	1.5	**0.64±0.04****	0.93±0.17	Jun B proto-oncogen
		15.6	**0.47±0.09****	**1.74±0.34***	
		B(a)P 30µM	1.13±0.36	**2.41±0.46***	

	MYC	1.5 15.6 *B(a)P* 30µM	0.98±0.04 0.92±0.03 1.07±0.07	0.96±0.16 **2.04±0.03***** **0.78±0.06***	V-Myc avian myelocytomatosis viral oncogene homolog
	TGFB2	1.5 15.6 *B(a)P* 30µM	1.13±0.19 1.33±1.06 0.10±0.77	**1.87±1.25** 0.86±0.49 **0.36±0.09****	Transforming growth factor. beta 2
DNA damage responsive	***TP53***	1.5 15.6 *B(a)P* 30µM	0.97±0.03 1.09±0.15 0.10±0.03	1.06±0.12 **1.50±0.22** 1.23±0.16	Tumor protein p53
	MDM2	1.5 15.6 *B(a)P* 30µM	1.00±0.001 1.11±0.04 0.10±0.03	1.1±0.20 **1.86±0.48** 1.13±0.21	Oncogene, E3 Ubiquitin Protein Ligase
	CDKN1A	1.5 15.6 *B(a)P* 30µM	0.85±0.09 0.96±0.00* 1.11±0.08	1.11±0.31 **2.17±0.48*** **7.33±2.02***	Cyclin-dependent kinase inhibitor 1A (p21.Cip 1)
	GADD45A	1.5 15.6 *B(a)P* 30µM	0.87±0.11 0.98±0.09 1.05±0.24	0.99±0.18 **1.52±0.27** **2.81±0.31***	Growth arrest and DNA-damage-inducible. alpha
	CHEK1	1.5 15.6 *B(a)P* 30µM	0.99±0.10 1.01±0.10 1.06±0.02	1.13±0.18 **0.68±0.26** **0.7±0.01*****	Checkpoint kinase 1
	ERCC4	1.5 15.6 *B(a)P* 30µM	1.09±0.04 1.03±0.04 1.06±0.04	1.07±0.12 1.25±0.08* **1.68±0.31**	Excision repair cross-complementing rodent repair deficiency. complementation group 4
Oxidative stress	***GCLC***	1.5 15.6	0.84±0.24 0.85±0.24	1.11±0.21 **2.14±0.20***	Glutamate-cysteine ligase. catalytic subunit

		B(a)P 30µM	0.96±0.16	**2.14±0.21***	
	GPX1	1.5	0.72±0.16	1.05±0.08	Glutathione peroxidase 1
		15.6	0.77±0.12	1.01±0.22	
		B(a)P 30µM	0.73±0.01	**1.67±0.19**	
	GSR	1.5	0.77±0.20	1.09±0.21	Glutathione reductase
		15.6	0.84±0.22	1.37±0.28	
		B(a)P 30µM	0.82±0.15	1.43±0.24	
	SOD1A	1.5	0.75±0.29	1.03±0.01*	Superoxide dismutase 1
		15.6	0.88±0.17	0.80±0.27	
		B(a)P 30µM	0.77±0.001***	1.15±0.21	
	CAT	1.5	0.74±0.22	0.73±0.28	Catalase
		15.6	1.10±0.00	**0.67±0.01*** **	
		B(a)P 30µM	0.88±0.07	**0.43±0.02*** **	
Apoptosis/ survival	***BAX***	1.5	0.94±0.01**	1.07±0.46	Apoptosis regulator BAX. BCL2 associated X protein
		15.6	1.03±0.03	1.12±0.05	
		B(a)P 30µM	0.97±0.16	1.35±0.05	
	BCL2	1.5	0.92±0.13	1.23±0.46	B-cell CLL/lymphoma 2
		15.6	1.03±0.11	1.16±0.05*	
		B(a)P 30µM	1.05±0.11	**0.10±0.05***	
	CASP3	1.5	0.93±0.09	0.97±0.02	Caspase 3. apoptosis-related cysteine peptidase
		15.6	0.89±0.07	1.05±0.05	
		B(a)P 30µM	1.13±0.1	1.03±0.17	
	CASP8	1.5	1.04±0.10	1.05±0.13	Caspase 8. apoptosis-related cysteine peptidase
		15.6	1.02±0.05	1.17±0.06	
		B(a)P 30µM	1.05±0.11	**2.11±0.14*** **	

CASP9	1.5	1.05±0.02	1.01±0.16	Caspase 9. apoptosis-related cysteine peptidase
	15.6	1.01±0.03	1.23±0.04*	
	B(a)P 30µM	1.03±0.08	0.90±0.06	
FAS	1.5	1.04±0.04	1.02±0.16	Fas (TNF receptor superfamily. member 6)
	15.6	0.95±0.02	1.08±0.20	
	B(a)P 30µM	1.15±0.07	**4.86±0.3****	

The results are expressed as relative mRNA expression normalized to the control group. Data are means ± SD of two independent experiments. Significant differences between Clay2 treated cells and the control group is indicated by *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-tailed Student's t-test comparison). Bold values indicate up or down-regulation of genes.

CAPÍTULO 5 / CHAPTER 5

Sara Maisanaba, Susana Aucejo, Ana M. Cameán, Ángeles Jos

EFFECTS OF TWO ORGANOMODIFIED CLAYS INTENDED TO FOOD CONTACT MATERIALS ON THE GENOMIC INSTABILITY AND GENE EXPRESSION OF HEPATOMA CELLS

Enviado a Toxicology Letters/ Sent to Toxicology Letters

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Manuscript Draft

Manuscript Number:

Title: Effects of two organomodified clays intended to food contact materials on the genomic instability and gene expression of hepatoma cells

Article Type: Full Length Article

Keywords: food contact material; organomodified clays; cytokinesis block micronucleus cytome assay; gene expression; qPCR

Corresponding Author: Mrs. Sara Maisanaba,

Corresponding Author's Institution:

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Maria Jorda-Beneyto; Ana M. Cameán; Angeles Jos

Abstract: Globally, food industries have made significant progress in order to increase the shelf-life of food products and have fewer economic losses. In this sense, the use of organomodified clays destined to be incorporated in polymer matrices play a novel role, leading to improved materials named nanocomposites with enhanced technological profiles. Due to the presence of these clays into the package, the safety of the consumers is a main concern. Cloisite®30B and Clay1 are two organomodified clays containing quaternary ammonium salts as modifiers, that can be potentially used to reinforce packaging polymers. Available toxicity data about these clays, specifically genotoxicity, is still limited and inconclusive in some aspects. Thus, the purpose of this work was to evaluate both clays ability to induce genomic instability through the cytokinesis block micronucleus cytome assay (CBMN) and for the first time, to the extent our knowledge, their influence in the modulation of several genes involved in genotoxicity and cell death mechanisms. Overall, no genotoxicity response was obtained in any case at the conditions tested. Similarly, significant gene deregulations were not observed on the genes selected. Nevertheless, further studies are highly needed to elucidate and increase the knowledge about the molecular mechanisms of clays toxicity.

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2. Surname (Last Name): Maisanaba
3. Date: 09-September-2015

4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title
Effects of two organomodified clays intended to food contact materials on the genomic instability and gene expression of hepatoma cells

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4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Sara Maisanaba

5. Manuscript Title
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Corresponding Author's Name: Sara Maisanaba

5. Manuscript Title
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Effects of two organomodified clays intended to food contact materials on the genomic instability and gene expression of hepatoma cells

Sara Maisanaba[a*], María Jordá-Beneyto[b], Ana M. Cameán[a], Ángeles Jos[a]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[b] Area of Packaging Materials and Systems, ITENE, C/Albert Einstein 1, 46980 Paterna, Valencia, Spain.

*Corresponding author:

Sara Maisanaba

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: saramh@us.es

Tel: +34 954 556762

Abstract

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Keywords: food contact material; organomodified clays; cytokinesis block micronucleus cytome assay; gene expression; qPCR.

1. Introduction

Food and beverage packaging is a large-scale business and, in recent years, a significant number of achievements have been made in order to increase the shelf-life of food products and have fewer economic losses. The use of natural substances to improve the packaging material properties largely refers to the introduction of fillers to generate a new kind of polymer. Clay minerals are within those new substances which have been used in the development of environmentally and ecologically friendly materials as sustainable solutions (Bordes et al., 2009; Hsu et al., 2012). Actually, clays are considered as the materials of the 21st century due to, among other reasons, their wide range of applications (Bergaya and Lagaly, 2013).

Clay minerals are layered substances consisting of sheets of silicate tetrahedra (SiO_4) and octahedra (containing Al, Mg, and Fe) (Zhu and Njuguna, 2014). Among the most common clays stand out the montmorillonite (Mt), an abundant clay in nature, derived from volcanic ash and rocks, that belongs to the smectite group, which consists of an octahedral sheet sandwiched by two opposing tetrahedral sheets (Leszczynska et al., 2007; Hatzigrigoriou and Papaspyrides, 2011; Theng, 2012). Mt is constituted of nanometer scale platelets of magnesium aluminumsilicate, which present 1nm thick and 100 to 500 nm in diameter, resulting in platelets of high aspect ratio (Uyama et al., 2003). This kind of clay presents the unique ability to incorporate various ions, so the surface can be either hydrophilic or hydrophobic depending on the charge and available solutes. Due to the relatively high cationic exchange capacity, high aspect ratio, and ease of expansion of the interlayer space, Mt can interact with a wide range of organic species, which is a critical property required for many applications, such as in the food packaging area (Zhu and Njuguna, 2014).

In this manner, the incorporation of Mt into the polymers as nanofillers (due to their layers thickness) generates polymer nanocomposites, defined as polymeric materials containing well-dispersed nanofillers which exhibit dramatic improvement in a range of characteristics, including mechanical properties, barrier properties, thermal stability, and flame retardancy, among others (Zhu and Njuguna, 2014). But it is important to highlight that clay aggregates must be exfoliated into single platelets and distributed homogeneously throughout the polymer phase to take full advantage of nanoclays high surface area (Sinha Ray and Okamoto, 2003; Sinha Ray and Bousima, 2005). Dispersion of clay layers into the polymer

is affected by mismatches between the hydrophobic/hydrophilic character of polymers and clays. Polymers are typically hydrophobic while clays are hydrophilic. In this sense, clays are often chemically modified with organic modifiers to render their surface more hydrophobic and improve their compatibility with polymers (Arora and Padua, 2010). For example, formation of nanocomposites with organoclays have shown pronounced improvements in the mechanical properties of various bio-polymers, even with a low level of filler loading (<5 wt%) and also excellent barrier properties against gases (e.g., O_2 and CO_2) and water vapor (Choudalakis and Gotsis, 2009; Rhim et al., 2013).

Quaternary ammonium salts are among the most used modifiers, giving improved compatible clays. Some of these organomodified clays are commercially available, such as Cloisite®30B (C30B) that contains methyl, tallow, bis-2-hydroxyethyl as modifier, and others are under development, such as Clay1, a modified clay with hexadecyltrimethyl-ammonium bromide, designed by the Technological Institute of Packaging, Transport and Logistic (ITENE, Valencia). Some technological advances are described by Lee et al. (2002) about a biodegradable polymer/Mt, where polybutylene succinate (PBST) and C30B were used as biodegradable polymer matrix and organomodified clay, respectively. The tensile properties of PBST/C30B nanocomposites with various contents of the nanoclays demonstrated a notable improvement compared to the raw polymer.

Although the technological profile of nanocomposites are well known, safety issues are also of importance, as modified novel clays can reach both humans and the environment. Available data on clays toxicity is still limited, but different authors have already described toxic effects induced by different organomodified clays, including C30B and Clay1. Cytotoxic effects were evaluated by different endpoints like neutral red uptake, MTS reduction assay and protein content in several model systems such as HepG2 (human hepatoma cell line), Caco-2 (human adenocarcinoma cell line) and HUVE cells (human vascular endothelium cell line). It was demonstrated a decrease in the cellular viability after C30B exposure in all of the biomarkers tested and almost absence of cytotoxic effects after Clay1 exposure for 24 and 48h, in a concentration range of 0-500 and 0-8 µg/mL of each clay, respectively (Maisanaba et al., 2013; Maisanaba et al., 2014; Houtman et al. 2014; Jordá-Beneyto et al. 2014; Maisanaba et al. 2015a). Moreover, C30B also induced genotoxic and mutagenic effects, determined by the Comet Assay and the Ames test, as well as, oxidative stress alterations *in vitro* (Maisanaba et al., 2013, 2015a). However, Clay1 only showed a mutagenic response in the bacterial assay,

in presence of external metabolic activation, but not DNA strand breaks or oxidative effects (Sharma et al., 2010; Maisanaba et al., 2013; Houtman et al., 2014; Maisanaba et al., 2015a). Maisanaba et al. (2015b) have recently reviewed the scientific literature on the toxicological evaluation of clay minerals. Although the data available are scarce in comparison to other compounds, cytotoxicity assays are the most abundant while mechanistic studies, such as genotoxicity, oxidative stress induction, inflammation, etc., are more limited.

Nowadays, a specific genotoxicity evaluation is required for new substances intended to be used as food contact materials by some regulatory and advisory bodies for authorization purposes (EFSA 2011, 2015) by means of a core set of tests. In this sense, the micronucleus test is one of the main genotoxicity tests proposed, but for modified clay minerals, in general, to the extend our knowledge, the information remains very scarce.

Therefore, due to the limited data about toxicity mechanisms of modified clays, the aim of our study was to analyze whether C30B and Clay1 had influence on genomic instability by evaluation of the formation of micronucleus (MNs), nuclear buds (NBUD), and nucleoplasmic bridges (NPB) in the human hepatoma cell line HepG2 by the Cytokinesis block micronucleus cytome assay (CBMN). Moreover, the alterations in the expression patterns of a several genes involved in genotoxicity mechanisms as well as cell death pathways by both clays were investigated by real-time quantitative PCR (qRT-PCR). Thus, the information obtained would allow to complete their toxicological assessment required to ensure their safety as food packaging materials.

2. Materials and Methods

2.1. Supplies and Chemicals

Minimum essential medium (MEM), TRIzol® reagent, foetal bovine serum (FBS), non-essential aminoacids (NEAA), penicillin/streptomycin, L-glutamine and trypsin were from Gibco (Biomol, Spain); phosphate buffered saline (PBS), cytochalasin B (Cyt-B), acridine orange (AO), dimethyl sulfoxide (DMSO [CAS 67-68-5]), etoposide (ET) [CAS 33419-42-0] and benzo(*a*)pyrene (B(*a*)P) [CAS 50-32-8], were obtained from Sigma–Aldrich (St. Louis, USA). High Capacity cDNA Archive Kit was from Applied Biosystems (Forest City, CA, USA Premix Ex Taq (Probe qPCR) (Takara-Clontech, France) and PrimeTime®Std qPCR assays (Integrated DNA Technologies (IDT), Madrid, Spain).

2.2. Modified clay and characterization

Cloisite®30B, were obtained from Southern Clay Products, INC. (modifier: methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium, concentration: 90 meq/100 g clay). Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) ($C_{19}H_{42}BrN$, 364.46 g/mol, 98%) was used for the development of Clay1 by cation exchange and was supplied by CymitQuimica S.L. (Spain). Both clays were characterized by thermogravimetric analysis (TGA), Fourier Transform InfraRed (FTIR) and X-ray diffraction (XRD) as described in previous works (Maisanaba et al., 2013; Jordá-Beneyto et al., 2014).

2.3. Clay test solutions

Test concentrations of clays were determined individually in previous experiments in order to avoid interferences with the method of measurement according to Maisanaba et al. (2013). The concentrations selected for the genotoxicity and gene expression assays were the highest ones that did not show cytotoxic effects and statistical differences versus the control. Thus, the maximum concentrations were 31.25 μg/mL for C30B and 8 μg/mL for Clay1, according to Maisanaba et al. (2013) and Houtman et al. (2014), respectively.

2.4. Cell culture

HepG2 cells (human hepatocellular carcinoma epithelial cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% of FBS (Gibco, New Zealand), 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin/ streptomycin (Gibco, New Zealand). Cells were grown at 37°C and 5% CO_2 in humidified atmosphere. Cells were used at passages between 5 and 12.

2.5. Cytokinesis block micronucleus assay (CBMN)

Prior to the treatment, the cells were seeded onto 25 cm^2 culture flask (1 000 000 cells/ plate) (all Corning Costar Corporation, New York, USA) and incubated for 24h to attach. Then, medium was replaced with fresh medium containing non-toxic concentrations of C30B (7.8, 15.65, 31.25 μg/mL) and Clay1 (2, 4, 8 μg/mL) during 24h, selected by previous cytotoxicity assays (Maisanaba et al., 2013; Houtman et al., 2014). B(*α*)P (30 μM) and ET (1 μg/mL) were used as positive controls.

The CBMN assay was performed according to Straser et al. (2011). After the 24h treatment, the cells were washed twice with 1x PBS buffer, the medium containing Cyt-B (3

µg/ml) was added and the cells were incubated for additional 26 h at 37°C and 5% CO2 in humidified atmosphere. Below, the cells were washed, trypsinized, incubated in a cold hypotonic solution (75 mM KCl) for 5 min and fixed with a mix of methanol/acetic (3:1; v/v) and formaldehyde 37%. The fixed cells were put on microscope slides and air dried, stained with AO (10 µg/ml) and examined under the fluorescence microscope (Olympus BX61, Japan). Micronucleus (MNs), nucleoplasmic bridges (NPBs) and nuclear buds (NBs) were counted in 1000 binucleated cells (BNC) per experimental point at 400x magnification according to the criteria published by Fenech (2000) and the OCDE 487 guideline (1997). The NDI was calculated using the formula [M1+ 2M2 + 3(M3 +M4)]/500, where M1, M2, M3 and M4 represent the number of cells with one to four nuclei, respectively (Fenech, 2000). The experiments were repeated three times independently.

2.6. mRNA expression analysis- Real-time quantitative PCR (QRT-PCR)

After the incubation, the cells were washed with 1x PBS and total RNA was isolated using TRIzol® reagent, according to the manufacturer's protocol with minor modifications described in Maisanaba et al (2015c). Briefly, glycogen (2 mg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at -20°C to precipitate. The RNA was transcribed to cDNA using 1 µg of total RNA and cDNA High Capacity Kit, according to the manufacturer's protocol (Applied Biosystem, USA). Gene expression was quantified with real-time quantitative PCR (LightCycler® 480 System, Roche Diagnostic, Spain) using Premix Ex Taq (Probe qPCR) (Takara-Clontech, France)and PrimeTime®Std qPCR assays (Integrated DNA Technologies (IDT), Madrid, Spain). The following PrimeTime®Std qPCR assays were used: *TP53* (tumor protein P53), NM_001276699; *MDM2* (Mdm2, 'MDM2 oncogene, E3 ubiquitin protein ligase), NM_002392; *CDKN1A* (cyclin-dependent kinase inhibitor 1A') NM_001220778; *GADD45A* ('growth arrest and DNA damage-inducible gene, alpha'), NM_001199741; *BAX* (BCL2-associated X protein), NM_004324; *BCL2* (B-cell CLL/lymphoma 2), NM_000633. These genes were selected based on previous studies that showed their deregulation by other clays (Maisanaba et al., 2015c, d). Amplification of *GAPDH* probe (Human Endogenous Controls, NM_002046) was performed as an internal control. The conditions for the PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data obtained from PrimeTime®Std qPCR assays were analyzed using an accurate quantification taking into account a control pattern (10x, 100x, 1000x, 10000x) and a normalization with the control group. The expression levels of target mRNAs were normalized to the GAPDH mRNA level. Two

independent experiments were performed each time in three parallels. An up-regulation and down-regulation of ≥ 1.5 and ≤ 0.7-fold change, respectively, compared to control was considered a positive response.

2.7. Statistical analysis

The statistical analyses were performed with GraphPad Instat software. In the CBMN assay Chi-square test was used to compare the number of MNs, NBUDs, and NPBs between the control group and Clay1 or C30B exposed cells. When calculating differences between samples for the gene expression profiles, statistical significance between treated group and control was determined by Two-tailed Student's t-test comparison. $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant.

3. Results

3.1. Genotoxicity of C30B and Clay1 in HepG2: Induction of micronuclei, nucleoplasmic bridges and nuclear buds by the CBMN

The genotoxic activity of C30B and Clay1 in HepG2 cells has been evaluated using the CBMN assay. Regarding to C30B, similar incidences compared to control group were observed for all the parameters and significant differences were not obtained in any case (Fig. 1).

On the other hand, after 24 h exposure, Clay1 induced only a statistically significant increase in the frequency of Buds (1.8-fold increase compared to control group) in BNCs at the highest concentration assayed (8 μg/mL); however, no changes in the frequency of the other parameters studied (MNed cells, MNs and NPBs) were observed (Fig. 2).

The positive control B(*a*)P (2.5 μM) induced statistically significant increases in frequencies of MNed cells (3.0 (C30B) and 2.7 (Clay1) -fold), MN (3.0 (C30B)and 2.6 (Clay1) -fold) and NBUDs (5.3 (C30B)and 4.9 (Clay1) -fold), but not NPBs. The another positive control, ET (1μg/mL), also induced statistical differences in the number of MNed cells (4.1 (C30B) and 3.8 (Clay1) -fold), MN (4.5 (C30B) and 4.0 (Clay1) -fold) and Buds (3.9 (C30B) and 3.3 (Clay1) - fold) (Fig.1 and 2). NDI was not affected in cells exposed to C30B or Clay1. Both positive controls, B(*a*)P and ET, decreased NDI (Fig.3a, b).

3.2. Effects of C30B and Clay1 on mRNA expression

The mRNa expression of a wide range of selected genes was analyzed after 4 and 24h of exposure to 3.125 and 31.25 C30B and, 0.8 and 8 µg/mL of Clay1 by quantitative real-time PCR (Table 1). The values presented in the Table 1 represent relative change in the gene expression of the selected genes compared to control group (untreated cells).

In HepG2 cells exposed to C30B and Clay1 for 4 and 24h, the mRNA levels of the genes involved in DNA damage response (*TP53*, *MDM2*, *CDKN1A*, *GADD45A*) did not show any ≥ 1.5 or ≤ 0.7-fold change compared to the control group. Therefore, genes are not considered to be deregulated although significant differences have been observed in the relative expression of all of them.

Moreover, no changes in the expression of selected apoptosis/survival responsive genes (*BAX* and *BCL2*) were observed in HepG2 cells exposed to C30B and Clay1 for 4 and 24h at the conditions tested, in spite of the significant results obtained.

4. Discussion

Toxic effects of clay minerals are of interest as it has been recently reviewed by Maisanaba et al. (2015b). This may be due to their increasing number of applications that can potentially increase human and environmental exposure. Moreover, the inclusion of modifiers in their structure leads to new compounds that can show a different toxicological profile in comparison to the raw material. Thus, a case by case toxicological evaluation is necessary.

In this study, C30B and Clay1 showed a similar pattern in the CBMN assay, and they did not altered the frequency of micronucleus at the conditions tested. However, both of them have shown genotoxic effects when other genotoxicity assays have been used (Sharma et al., 2010; Maisanaba et al., 2013; Houtman et al., 2014; Maisanaba et al., 2015a). Actually, genotoxicity testing requires the use of various assays that covers different genetic endpoints. Thus, EFSA (2011) recommends the use of the following two *in vitro* tests as the first step in testing: a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test. The first one covers gene mutations and the second one covers both structural and numerical chromosome aberrations. Taking into account that both C30B and Clay1 induced mutagenic effects by the Ames test (Maisanaba et al., 2015a) they can be considered genotoxic *in vitro*. Other clays, however, show a different profile. The raw clay (Mt, Cloisite®Na^+ or CNa^+), did not

show any effect on the bacterial assay (Maisanaba et al., 2015a) but it induced micronucleus (MNs) and important changes at the molecular level in HepG2 cells (Maisanaba et al., 2015c). The same authors also investigated the genotoxicity of Clay2, other organomodified clay similar to Clay1 but including also acetylcholine chloride as modifier. This one was not mutagenic by the Ames test (Maisanaba et al., 2015a), but on the contrary it induced important increases in the frequencies of MNs and other nuclear anomalies such as buds (Buds) and bridges (NPBs) (Maisanaba et al., 2015d).

Regarding to the effects of both clays on the RNAm levels of selected genes, in any case the change was large enough to consider their expression deregulated. However, the pattern was mainly a significant reduction in the values, and only 3.125 µg/L C30B after 24h induced *BAX* expression values >1. Nevertheless, the response of the positive control (B(*a*)P) was similar to previous studies performed with CNa^+ (Maisanaba et al., 2015c) and Clay2 (Maisanaba et al., 2015d). Therefore, all these findings suggested that neither C30B nor Clay1 induced changes in the expression of the genes studied. Other clays, however, showed upregulations of *TP53*, *MDM2* (Clay2), *CDKN1A*, *GADD45A* (Clay2 and CNa^+) and *BCL2* (CNa^+) (Maisanaba et al., 2015c, d).

Among the damage responsive genes, the tumor suppressor gen *P53* is one of the most important and studied genes. It activates several genes, which results in the arrest of the cellular cycle and cellular repair or apoptosis. Many are the signals that activate *P53* function including: DNA damage by gamma or ultraviolet radiation and chemical agents and hypoxia, among others. The final outcome of the different mechanisms of action of *P53* is to maintain the genomic stability of the cell (Mendoza-Rodríguez and Cerbón, 2001). *MDM2* is an important negative regulator of the *P53* tumor suppressor gene. Thus, increased levels of *MDM2* would inactivate the apoptotic and cell cycle arrest functions of *P53*, as do deletion or mutation of *P53* (Iwakuma and Lozano, 2003). *CDKN1A* and *GADD45A* are *TP53* downstream regulated genes. *CDKN1A* encodes a potent cyclin-dependent kinase inhibitor (p21) and functions as a regulator of cell cycle progression at G1 (Cazzalini et al., 2010). The role of *GADD45A* is to control the cell cycle G2-M checkpoint, the DNA repair process and apoptosis (Wang et al., 1999). Regarding to apoptosis responsive genes, *BCL2* is an anti-apoptoptic gene that confers negative control in the pathway of cellular suicide machinery. *BAX*, on the other hand, is a pro-apoptotic gene that promotes cell death (Basu and Haldar, 1998). None of them showed an altered expression in this case.

Taking into account the results presented in this study, it is observed that different clays showed different effects, maybe due to the modifiers they contain, or to the concentrations used. It is therefore justified the need for a comprehensive and detailed assessment of each clay, case by case.

5. Conclusion

The organomodied clays C30B and Clay1 did not show notable genotoxic effects by means of the CBMN assay under the conditions tested. Moreover, they did not deregulate the RNAm expression of the DNA damage response genes (*TP53*, *MDM2*, *CDKN1A* and *GADD45A) n*or apoptosis/survival genes *(BAX* and *BCL2)* genes. Further studies would be required to elucidate the mechanisms involved in their reported toxic effects previously to their use as polymer fillers.

Acknowledgements

The authors wish to thank Junta de Andalucía (AGR5969) for the financial support and Floriana Mandracchia for her help in the lab. Sara Maisanaba also gratefully acknowledges Junta de Andalucía for her predoctoral grant associated to the AGR5969 project.

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Figure captions

Figure 1. Number of binucleated cells with a) micronucleated cells (MNed cells), b) micronuclei (MN), c) nuclear bridges (NPBs) and d) cells with nuclear buds (NBUDs) per 1000 binucleated HepG2 cells after the exposure to C30B (0, 7.8, 15.6 and 31.25 μg/mL) for 24 h. The number of MNed cells shows the number of cells containing MN, while MN shows the overall number of MN as some cells may contain more than one MN. Benzo(*a*)pyrene (B(*a*)P; 2.5 μM) and Etoposide (ET; 1 μg/mL) were used as positive controls. Significant differences between treated cells and the control group is indicated by **P* < 0.05, ***P* < 0.01.

Figure 2. Number of binucleated cells with a) micronucleated cells (MNed cells), b) micronuclei (MN), c) nuclear bridges (NPBs) and d) cells with nuclear buds (NBUDs) per 1000 binucleated HepG2 cells after the exposure to Clay1 (0, 2, 4 and 8 μg/mL) for 24 h. The number of MNed cells shows the number of cells containing MN, while MN shows the overall number of MN as some cells may contain more than one MN. Benzo(*a*)pyrene (B(*a*)P; 2.5 μM) and Etoposide (ET; 1 μg/mL) were used as positive controls. Significant differences between treated cells and the control group is indicated by **P* < 0.05, ***P* < 0.01,

Figure 3. The influence of a) C30B (0, 7.8, 15.6 and 31.25 μg/mL) and b) Clay1 (0, 2, 4 and 8 μg/mL) on the nuclear division index (NDI) of HepG2 cells after 24h exposure. Benzo(*a*)pyrene (B(*a*)P; 2.5 μM) and Etoposide (ET; 1 μg/mL) were used as positive controls. Significant differences between treated cells and the control group is indicated by **P* < 0.05, ***P*<0.01.

Table caption

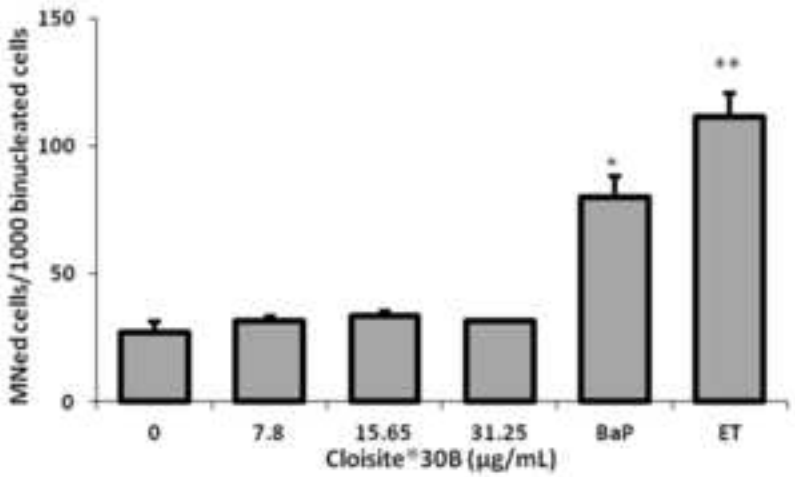
Table 1. Effect of C30B and Clay1 on the expression of mRNAs of selected genes involved in DNA damage response and apoptosis/survival in HepG2 cells. The cells were exposed to C30B (0, 3.125 and 31.25 μg/mL) or Clay1 (0, 0.8 and 8 μg/mL) for 4 and 24h. Benzo(*a*)pyrene (B(*a*)P; 30μM) was used as positive control. Statistical significance between treated and control group was determined by Two-tailed Student's t-test comparison. *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***) were considered as statistically significant; an up-regulation and down-regulation of ≥ 1.5 and ≤ 0.7-fold change, respectively, compared to control was considered a positive response (marked in bold).

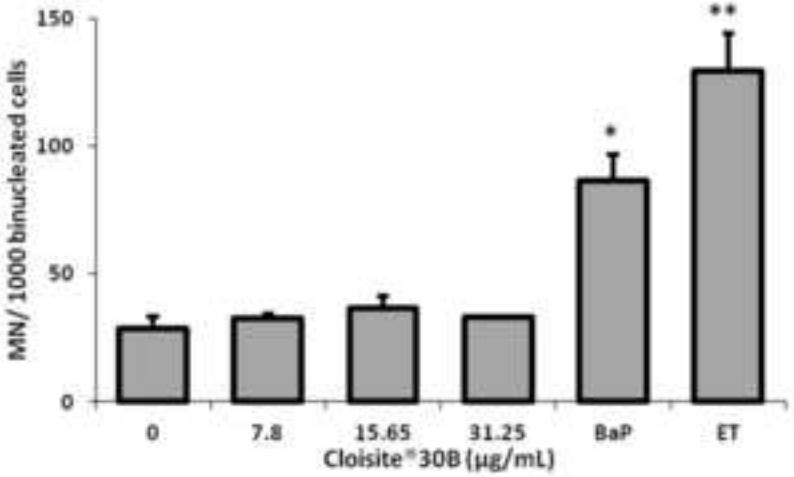
Table

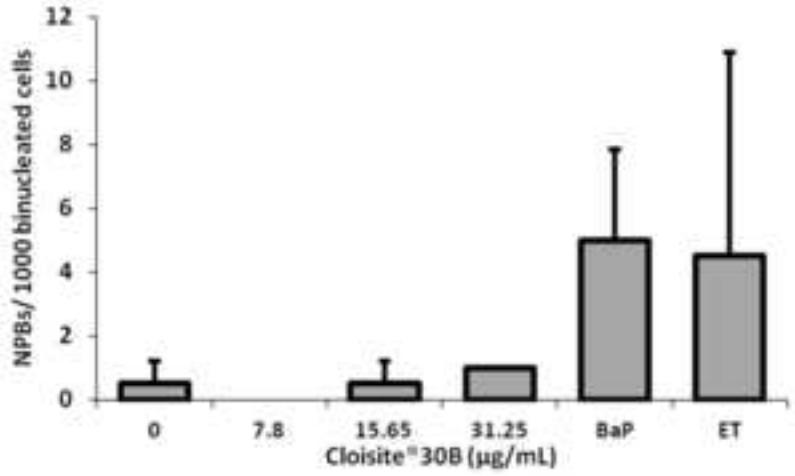
	Clay1				Cloisite®30B				Benzo(*a*)pyrene (30µM)	
	4h		24h		4h		24h		4h	24h
	0.8µg/µL	8µg/µL	0.8µg/µL	8µg/µL	3.125µg/µL	31.25µg/µL	3.125µg/µL	31.25µg/µL		
TP53	0.83±0.04**	0.79±0.04**	0.86±0.02***	1.02±0.21	0.78±0.06	0.78±0.03	0.89±0.11	1.03±0.21	0.87±0.10	1.30±0.33
MDM2	0.81±0.01***	0.80±0.07**	0.93±0.00***	0.84±0.18	0.78±0.05**	0.77±0.05**	0.91±0.01***	0.83±0.21	0.95±0.05	1.00±0.08
CDKN1A	0.78±0.03***	0.83±0.11	0.73±0.05*	0.74±0.05	0.84±0.07***	0.87±0.12***	0.80±0.06**	0.81±0.28	1.07±0.05	**4.61±1.33****
GADD45A	0.76±0.04***	0.83±0.10	0.74±0.03	0.76±0.10	0.90±0.08***	0.99±0.43**	0.86±0.02***	0.94±0.33	0.97±0.04	**2.74±0.42****
BAX	1.06±0.08	1.02±0.06	1.02±0.14	0.98±0.02	1.10±0.09	0.95±0.09	1.14±0.07*	0.90±0.13	0.85±0.06**	0.90±0.15
BCL2	0.78±0.13*	0.76±0.15*	0.64±0.12	1.12±0.15	0.98±0.26	0.92±0.19**	0.64±0.14	1.26±0.23	1.29±0.33	**0.30±0.17****

The results are expressed as relative mRNA expression normalized to the control group. Data are means ± SD of three independent experiments. Significant differences between Clay1 and Cloisite®30B treated cells and the control group is indicated by *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two-tailed Student's t-test comparison). Bold values indicate up or down-regulation of genes.

Figure


MNed cells/1000 binucleated cells
150
100
50
0
*
**
0
7.8
15.65
31.25
BaP
ET
Cloisite®30B (µg/mL)


MN/ 1000 binucleated cells
150
100
50
0
*
**
0
7.8
15.65
31.25
BaP
ET
Cloisite®30B (µg/mL)


NPBs/ 1000 binucleated cells
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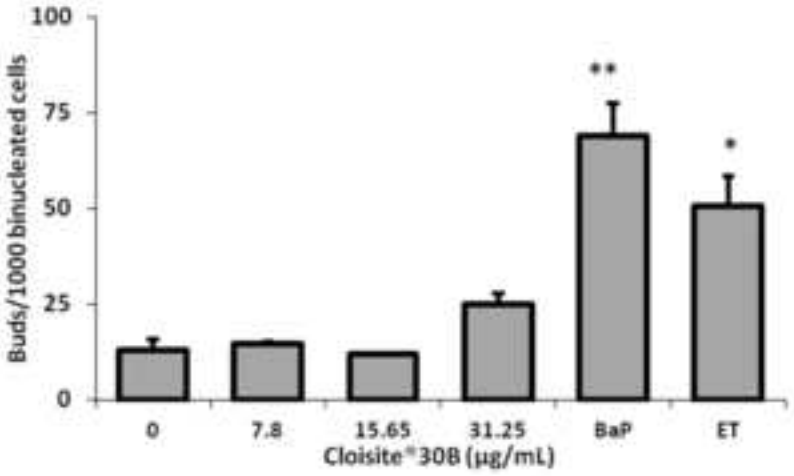
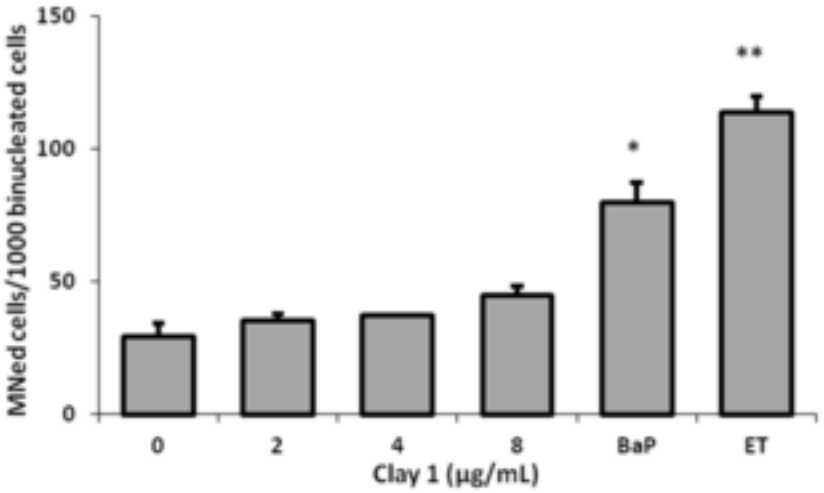
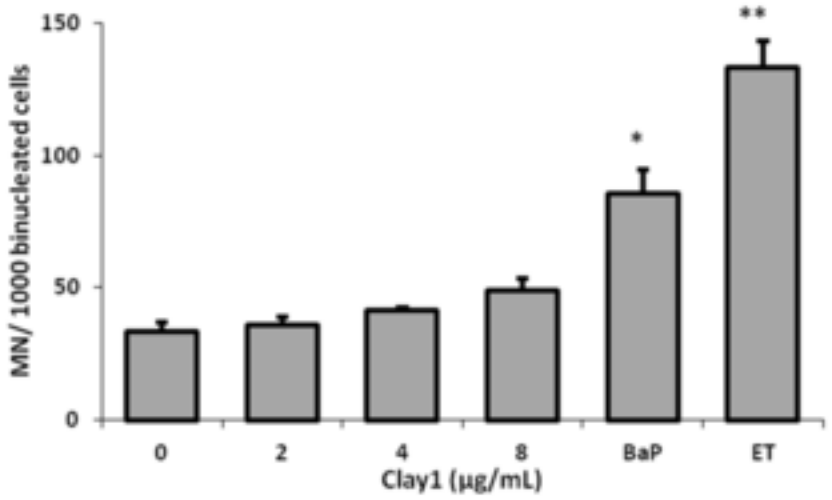
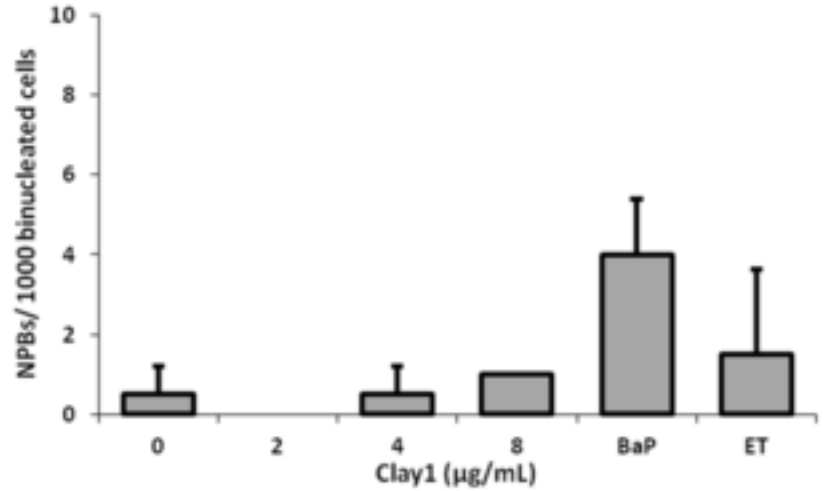
10
8
6
4
2
0
0
7.8
15.65
31.25
BaP
ET
Cloisite®30B (µg/mL)

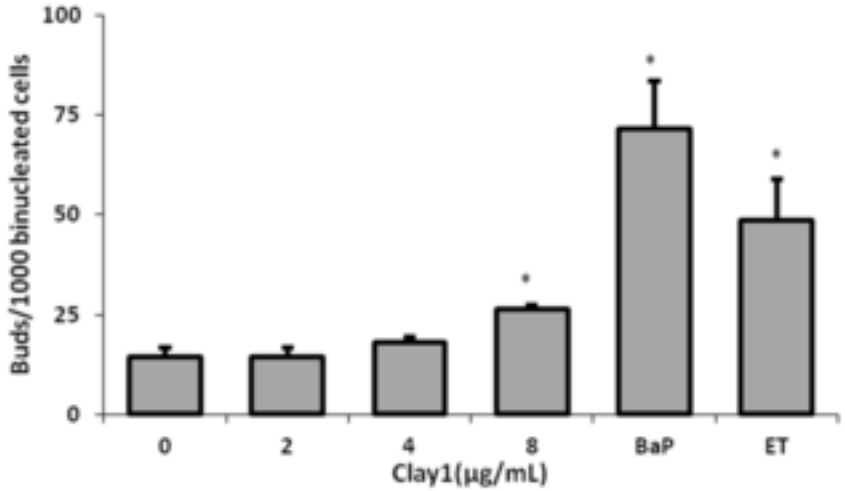

Buds/1000 binucleated cells
100
75
50
25
0
**
*
0
7.8
15.65
31.25
BaP
ET
Cloisite®30B (µg/mL)

Figure

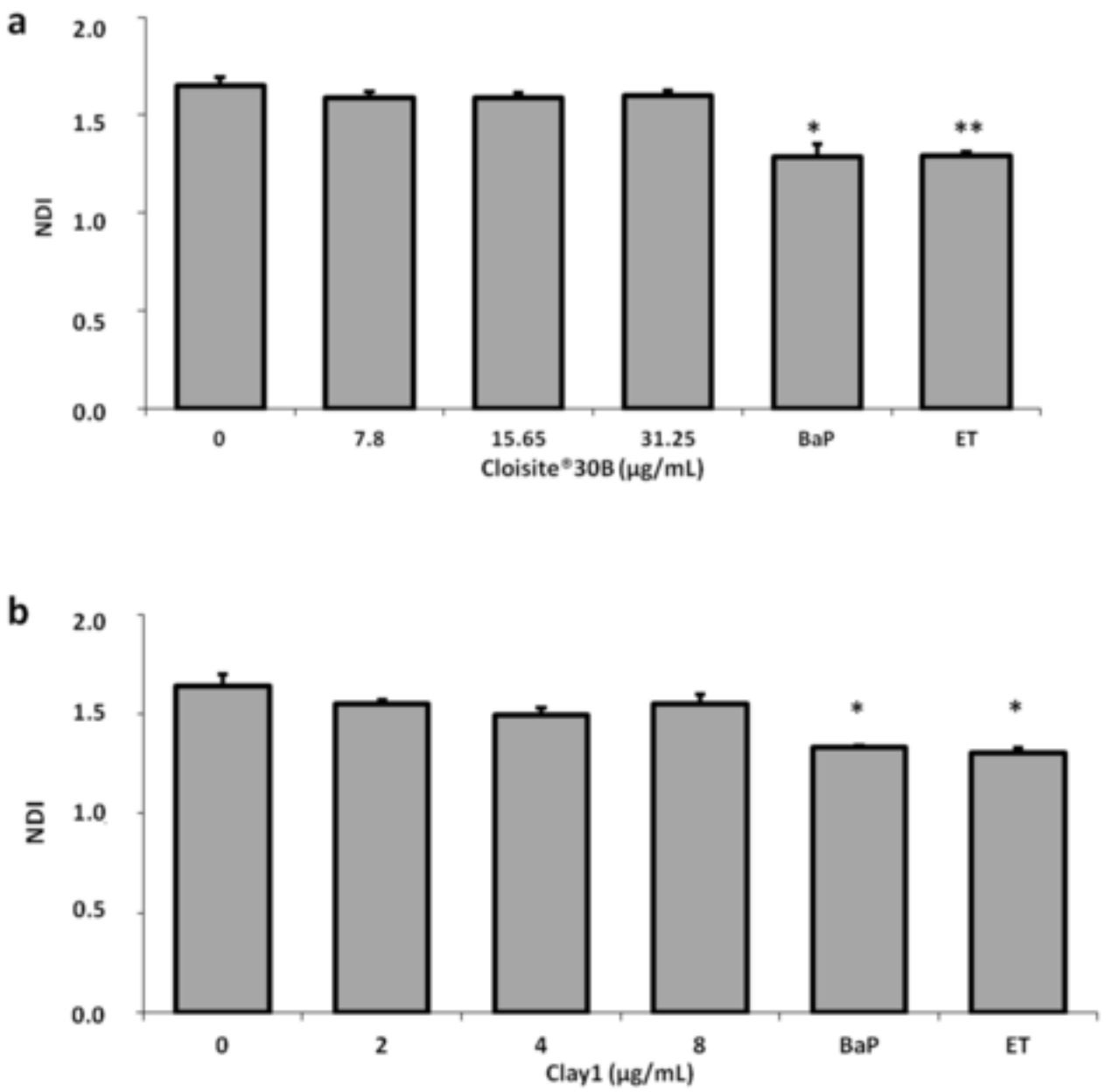

MNed cells/1000 binucleated cells
150
100
50
0
*
**
0
2
4
8
BaP
ET
Clay 1 (µg/mL)


MN/ 1000 binucleated cells
150
100
50
0
*
**
0
2
4
8
BaP
ET
Clay1 (µg/mL)


NPBs/ 1000 binucleated cells
10
8
6
4
2
0
0
2
4
8
BaP
ET
Clay1 (µg/mL)


Buds/1000 binucleated cells
100
75
50
25
0
*
*
*
0
2
4
8
BaP
ET
Clay1(µg/mL)

Figure


a
NDI
2.0
1.5
1.0
0.5
0.0
*
**
0
7.8
15.65
31.25
BaP
ET
Cloisite®30B (µg/mL)
b
NDI
2.0
1.5
1.0
0.5
0.0
*
*
0
2
4
8
BaP
ET
Clay1 (µg/mL)

CAPÍTULO 6 / CHAPTER 6

Sara Maisanaba, Daniel Gutiérrez-Praena, Silvia Pichardo, F. Javier Moreno, María Jordá, Ana M. Cameán, Susana Aucejo, Ángeles Jos

TOXIC EFFECTS OF A MODIFIED MONTMORILLONITE CLAY ON THE HUMAN INTESTINAL CELL LINE CACO-2

Journal of Applied Toxicology 34, 714-725, ***2014***

SPi | Journal Code: J A T | Article ID: 2 9 4 5 | Dispatch: 02.10.13 | CE: | No. of Pages: 12 | ME:

Research Article

Journal of Applied Toxicology

Received: 26 July 2013, Revised: 2 September 2013, Accepted: 9 September 2013 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jat.2945

Toxic effects of a modified montmorillonite clay on the human intestinal cell line Caco-2

Sara Maisanaba[a], Daniel Gutiérrez-Praena[a], Silvia Pichardo[a]*, F. Javier Moreno[b], María Jordá[c], Ana M. Cameán[a], Susana Aucejo[c] and Ángeles Jos[a]

ABSTRACT: The incorporation of the natural mineral clay montmorillonite into polymeric systems enhances their barrier properties as well as their thermal and mechanical resistance, making them suitable for a wide range of industrial applications, e.g., in the food industry. Considering humans could easily be exposed to these clays due to migration into food, toxicological and health effects of clay exposure should be studied. In the present work, the cytotoxic effects induced by two different clays (the unmodified clay Cloisite® Na^+, and the organically modified Cloisite® 30B) on Caco-2 cells were studied after 24 and 48 h of exposure. The basal cytotoxicity endpoints assessed were total protein content, neutral red uptake and a tetrazolium salt reduction. Our results showed that only Cloisite® 30B induced toxic effects. Therefore, the effects of subcytotoxic concentrations of this clay on the generation of intracellular reactive oxygen species, glutathione content and DNA damage (comet assay) were investigated. Results indicate that oxidative stress may be implicated in the toxicity induced by Cloisite® 30B, in regards of the increases in intracellular reactive oxygen species production and glutathione content at the highest concentration assayed, while no damage was observed in DNA. The most remarkable morphological alterations observed were dilated cisternae edge in the Golgi apparatus and nucleolar segregation, suggesting impairment in the secretory functions, which could be related to inhibition in the synthesis of proteins. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: clays; Cloisite®; cytotoxicity; oxidative stress; morphology; cell culture

Introduction

Modern food packaging has made great advances as result of global trends and consumer preferences, which are oriented to obtain improved food quality and safety. Technological innovation in this field comprises the addition of different compounds to the polymeric matrix to obtain improved packaging materials (Silvestre *et al*., 2011). One of the preferred compounds used for these applications are clays, such as montmorillonite, which acquire nanoscale dimensions when incorporated to polymers, resulting in nanocomposites (Rhim and Ng, 2007). These nanocomposites are among the first polymer nanomaterials to emerge on the market as improved materials for food packaging (Silvestre *et al*., 2011). Montmorillonite is a hydrated aluminasilicate layered clay consisting of an edge-shared octahedral sheet of aluminum hydroxide between two silica tetrahedral layers, which is a relatively cheap and widely available natural clay derived from volcanic ash/rocks (Paiva *et al*., 2008). Layered clays have been widely studied and are of particular interest due to successful incorporation into composite materials and the advantageous properties that reinforced materials exhibit, which are attributed to their high aspect ratio (Rhim and Ng, 2007). This is particularly important when high barrier properties are needed. Several methods have been used to obtain a homogeneous distribution of clay in the matrix and exfoliation of the clay, which led to a nanoclay structure of the montmorillonite into the polymer (Silvestre *et al*., 2011).

Although beneficial effects of nanotechnologies are generally well described, the possible toxicological effects and impacts of nanocomposites on human health have received little attention so far. There are still many important knowledge gaps in human risk assessment concerning nanofood-related products, that if resolved will bring the risk assessment of nanocomposites, following oral exposure, a step forward (Bouwmeester *et al*., 2009). In this sense, indirect contamination of food can be expected when nanoparticles or nanotechnological devices are incorporated in to packaging materials or storage containers to lengthen the storage time while keeping the products fresh. Considering that migration of these kinds of materials from the packaging to food could occur, their safety in the food industry should be studied (EFSA, 2011). In this sense, despite great research into using montmorillonite in food packaging and other applications, the toxic effects on humans remains limited; therefore the need to generate better understanding about its potential negative impacts on biological systems urges (Bouwmeester *et al*., 2009). However, different experiments have assessed the toxicity of montmorillonite for other applications in the food industry. In this concern, Wang *et al*. (2005) have

*Correspondence to: Silvia Pichardo Sánchez, Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville, Spain. E-mail: spichardo@us.es

[a]Department of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville, Spain

[b]Department of Cellular Biology, Faculty of Biology, University of Seville, Avda. Reina Mercedes s/n, 41012 Seville, Spain

[c]Department of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain

reported the relative safety of calcium montmorillonite in human subjects when added to the diet to diminish exposure and health risk from aflatoxin-contaminated food. Moreover, the safety of calcium montmorillonite used for this purpose have been also confirmed in multiple animal species as well as in humans, and even in long-term animal studies (Afriyie-Gyawu *et al.*, 2005; Phillips *et al.*, 2008; Robinson *et al.*, 2012). Additionally, calcium and sodium montmorillonite showed no toxicity in pregnant rats at levels in the diet up to 2% w/w (Wiles *et al.*, 2004).

It is clear that cell culture-based toxicity tests are of interest, having the potential to screen samples for a biochemical response, while retaining the ability to detect more general cytotoxicity endpoints (Puerto *et al.*, 2009). Hence, the Caco-2 cell line is a commonly used enterocytic model, established from human colon carcinoma, which in culture undergoes a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing morphological and functional characteristics of the mature enterocyte (Sambuy *et al.*, 2005). Plenty of experiments have been already conducted in this cell line evidencing its accuracy as an experimental model to assess toxic effects of substances that could target the gastrointestinal system (Da Violante *et al.*, 2002; Jos *et al.*, 2009a, 2009b; Gutiérrez-Praena *et al.*, 2012; Loh *et al.*, 2012; Pichardo *et al.*, 2012).

Taking into account all this background information, the present work aims to assess the biochemical and morphological alteration caused by two clays (the unmodified clay Cloisite® Na+, and the organically modified Cloisite® 30B) on the human colon cell line Caco-2 after 24 and 48 h of exposure. First, the cytotoxic response of exposure to clays was studied by three different endpoints: total protein content (PC), neutral red uptake (NR) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) reduction. Moreover, the alteration in the oxidative status, DNA damage as well as the implication of glutathione (GSH) in the toxic mechanism of clays has been assessed. Finally, morphological alterations were observed by light and electron microscopy to explore the intracellular targets of these clays.

Materials and Methods

Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). The protein reagent assay was obtained from BioRad (Spain).

Clay Materials and Characterization

Unmodified montmorillonite (Cloisite® Na^+) and the organically modified one (Cloisite® 30B) were obtained from Southern Clay Products, INC. (modifier: methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium, concentration: 90 meq 100 g^{-1} clay). Both clays were characterized by termogravimetric analysis, X-ray diffraction and particle size distribution as described in Maisanaba *et al.* (2013). Termogravimetric analysis showed a large loss of mass for the Cloisite® 30B in the range 200–500 °C whereas Cloisite® Na^+ did not present any change in the same range of temperature. The X-ray diffraction analysis for both materials showed the typical pattern associated with a montmorillonite material. Finally, the particle size distribution analysis showed that the particle size of Cloisite® 30B is lower than those of Cloisite® Na^+ with both of them at the microscale (Maisanaba *et al.*, 2013).

Model System

The Caco-2 cell line derived from a human colon carcinoma (ATCC® HTB-37, passages 29–48) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 50 μg ml^{-1} gentamicin, 1.25 μg ml^{-1} fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were grown near confluence in 75 cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer hemocytometer and viability was determined by exclusion of Trypan Blue. The Caco-2 cells were plated at a density of 7.5×10^5 cells ml^{-1} and incubated at 37 °C for 24 h until confluence was achieved to perform the experiments.

Cytotoxicity Assays

The test concentrations of both clays were determined individually in previous experiments to avoid interferences with the method of measurement. For this reason the absorbance of clay solutions (1000 μg ml^{-1} and serial half dilutions) were measured at 0, 24 and 48 h. The highest concentrations selected were the ones that did not show statistical differences versus the control. Stock solutions were 1000 μg ml^{-1} for both clays. Three sonication steps of 10 s each one at an amplitude of 40% were performed used an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentration. From the initial solution, serial dilutions in medium were prepared (0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5 and 125 μg ml^{-1} for Cloisite® Na^+; 3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 μg ml^{-1} for Cloisite® 30B). The culture medium without clay was used as the control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37 °C for 24 and 48 h. The basal cytotoxicity endpoints were PC, supravital dye NR cellular uptake, and MTS.

PC is a very useful endpoint to assess cytotoxicity, as it gives data about cell damage independence of the toxic mechanism involved (Pichardo *et al.*, 2007). PC was quantified *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, to determine the total cell number present in the culture. The culture medium was replaced by 200 μl NaOH 0.1 M and after 2 h incubation at 37 °C, 180 μl was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm (Infinite M200; Tecan, Austria).

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, NR in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. The NR can be extracted from lysosomes for quantitative measurement of cells viability and cytotoxicity of xenobiotics. Culture medium was replaced by 100 μl medium without serum containing 10 mg ml^{-1} NR. The 96-well plate with the NR-containing medium was

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returned to the incubator for another 3 h to allow the uptake of NR into the lysosomes of viable, intact cells. Thereafter, the medium was removed and cells were fixed for 1 min with a formaldehyde–$CaCl_2$ solution. By adding 0.2 ml of acetic acid–ethanol solution to the wells, the NR absorbed by the cells was extracted, brought into solution and quantified at 540 nm (Infinite M200; Tecan).

MTS reduction is carried out by dehydrogenase enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop *et al.* (1991). The MTS tetrazolium compound added to the medium is bioreduced by cells seeded in the 96-well plate into a colored formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm (Infinite M200; Tecan) after 2 h of incubation in the dark.

Oxidative Stress Assays

Considering that only Cloisite® 30B showed remarkable cytotoxic effects, it was chosen to perform further studies. The concentrations used in these assays were calculated based on the cytotoxicity study previously performed. The most sensitive endpoint was PC; therefore, the mean effective concentration (EC_{50}) value obtained for this endpoint at 24 h, 40 ± 3.4 μg ml^{-1}, was chosen as the higher exposure concentration for the oxidative stress studies in the Caco-2 cell line, along with fractions $EC_{50}/2$ and $EC_{50}/4$, and the concentrations used here for Cloisite® 30B were 0, 10, 20 and 40 μg ml^{-1}.

After replacing the previous medium, the exposure solutions were added to the cells, and incubated at 37 °C for 24 h. Culture medium without clay was used as a control group. The oxidative stress endpoints measured were reactive oxygen species (ROS) content and GSH levels.

The production of ROS was assessed in 96-well plates using the dichlorofluorescein (DCF) assay. The probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Invitrogen) readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent compound (DCFH), which is rapidly oxidized in the presence of ROS to the highly fluorescent DCF. Specifically, cells were incubated with 200 μl 20 μM DCFH-DA in culture medium at 37 °C for 30 min, and then washed with phosphate-buffered saline (PBS) and resuspended in 200 μl of PBS. The formation of the fluorescence-oxidized derivative of DCF-DA was monitored at an emission wavelength of 535 nm and excitation wavelength of 485 nm (Infinite M200; Tecan). ROS production was expressed as fluorescence arbitrary units (Pichardo *et al.*, 2012).

GSH content in cells was evaluated in 96-well plates by reaction with the fluorescent probe monochlorobimane (Molecular Probes, Invitrogen) (Jos *et al.*, 2009a, 2009b). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme GST. After cell exposure to the clay, medium was discarded and cells were incubated at 37 °C for 20 min in the presence of 40 μM monochlorobimane. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Infinite M200; Tecan) at excitation/emission wavelengths 380/460. Results were expressed as arbitrary units.

Comet Assay

The comet assay was performed to detect DNA strand breaks. Caco-2 cells were seeded on to 12-well tissue culture-treated plates (Corning Costar Corporation, New York, USA). Approximately 3.5×10^5 cells in each well were exposed with a different concentrations of Cloisite® 30B (0, 10, 20 or 40 μg ml^{-1}) for 24 and 48 h.

To monitor the ongoing process of the assay, a negative control (cells treated with medium without fetal calf serum) and a positive control (cells treated with a solution of 100 μM H_2O_2) were included. After treatments, cells were washed and detached in PBS. The comet assay was applied as previously described by Collins *et al.* (1997) with modifications (Corcuera *et al.*, 2011). Briefly, cells were resuspended in PBS at a concentration of 2.5×10^6 cells ml^{-1}. This suspension were mixed with 1% low melting point agarose and placed on a microscope slide. Once the gels had become solid, the slides were dipped into lysis solution at 4 °C. All nucleotides were denatured in a high pH buffer (0.3 M NaOH, 1 mM EDTA, pH 13). Electrophoresis was carried out at approximately at 25 V (300 mA) and the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold nuclei acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20× objective) coupled via a CCD camera to an image analysis system (DP controller–DP manager). Images of randomly selected nuclei (≥ 100) per experimental point were analyzed with the image analysis software (Comet Assay IV; Perceptive Instruments, UK). The results from four independent experiments are expressed as percentage of tail DNA and are shown as box plots.

Morphology

Cells were exposed to two different concentrations of Cloisite® 30B, 10, 20 and 40 μg ml^{-1}, during 24 and 48 h of exposure. Afterwards, cultured cells were fixed directly in the cell culture dish in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4 °C. They were all postfixed in 1% osmium tetroxide for 60 min at 4 °C. Subsequently, cells were scraped and pelleted by centrifugation. Samples were washed in 0.1 M cacodylate buffer, pH 7.3. Samples were dehydrated in ethanol at progressively higher concentrations and embedded in Epon (epoxy embedding medium). Toluidine blue-stained semithin sections (0.5 μm thick) used as controls were viewed in a Leitz (Aristoplan) light microscope. The presence of lipids was confirmed by Sudan III cytochemical technique.

Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope. The cell growth and development of morphology damage was checked using a Leica DMIL inverted microscope by phase contrast and photographed using a Leica DC 100 camera.

Calculations and Statistical Analysis

All experiments were performed at least three times and in duplicate per concentration. Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $P < 0.05$.

EC_{50} values (mean effective concentration, concentration that modified each biomarker by 50%, positive or negative, in comparison with appropriate untreated controls) were determined by probit analysis.

Results

Results of the Cytotoxicity Assays

Caco-2 cells exposed to Cloisite® Na^+ showed no significant changes in PC after both exposure times at any concentration assayed (Fig. 1a). Similarly, NR uptake assay also indicated no significant alterations at 24 and 48 h (Fig. 1b). MTS reduction performed by cells exposed to Cloisite® Na^+ also remained unaltered with respect to the control cells after 24 h, whereas after 48 h significant reductions were observed from the concentration of 31.25 μg ml^{-1} Cloisite® Na^+ (Fig. 1c).

On the other hand, Caco-2 cells exposed to Cloisite® 30B underwent a concentration and time-dependent decrease in PC. Significant reductions in cell viability were shown from the concentrations of 31.25 μg ml^{-1} Cloisite® 30B in cells exposed for 24 h and from the concentration of 15.63 μg ml^{-1} Cloisite® 30B after 48 h of exposure (Fig. 2a). In addition, considering the EC_{50} values obtained, toxic effects were higher in the longest exposure, being 40 ± 3 μg ml^{-1} for 24 h and 34 ± 6 μg ml^{-1} for 48 h. However, this increase was not statistically different. The remaining cell viability of 5% was recorded after 24 h and 48 h of exposure to the highest concentration tested (250 μg ml^{-1}). NR uptake assay also indicated a reduction in cell viability. These decreases were significantly different from the control group at the concentration 62.50 μg ml^{-1} at 24 h, and from 31.25 μg ml^{-1} in cells exposed for 48 h (Fig. 2b). Moreover, significant differences were observed for the EC_{50} values in the NR uptake assay, being lower in the longest exposure time (50 ± 2 μg ml^{-1}

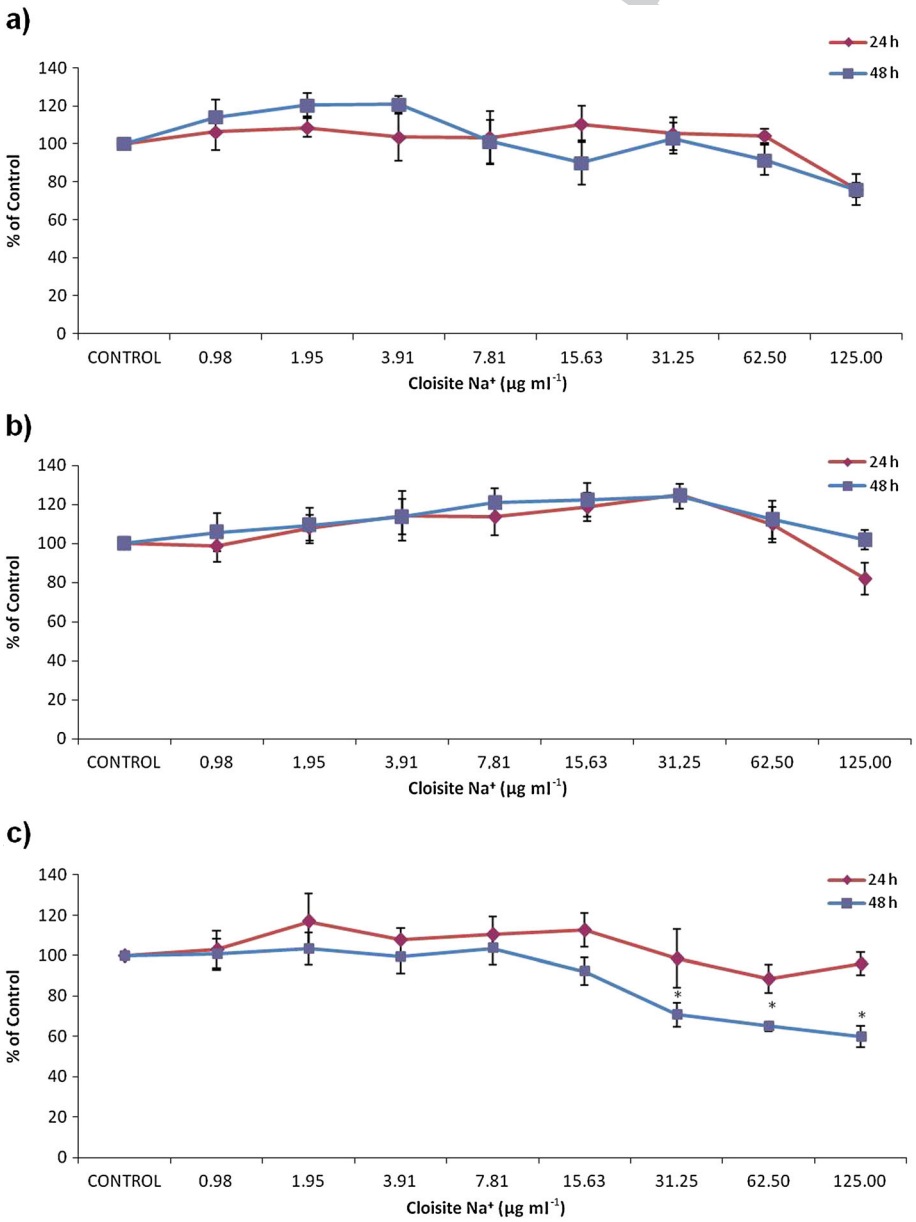

Figure 1. Protein content (a); neutral red uptake (b); and reduction of tetrazolium salt (c) of Caco-2 cells after 24 h and 48 h of exposure to 0–125 μg ml^{-1} Cloisite® Na^+. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.05$).

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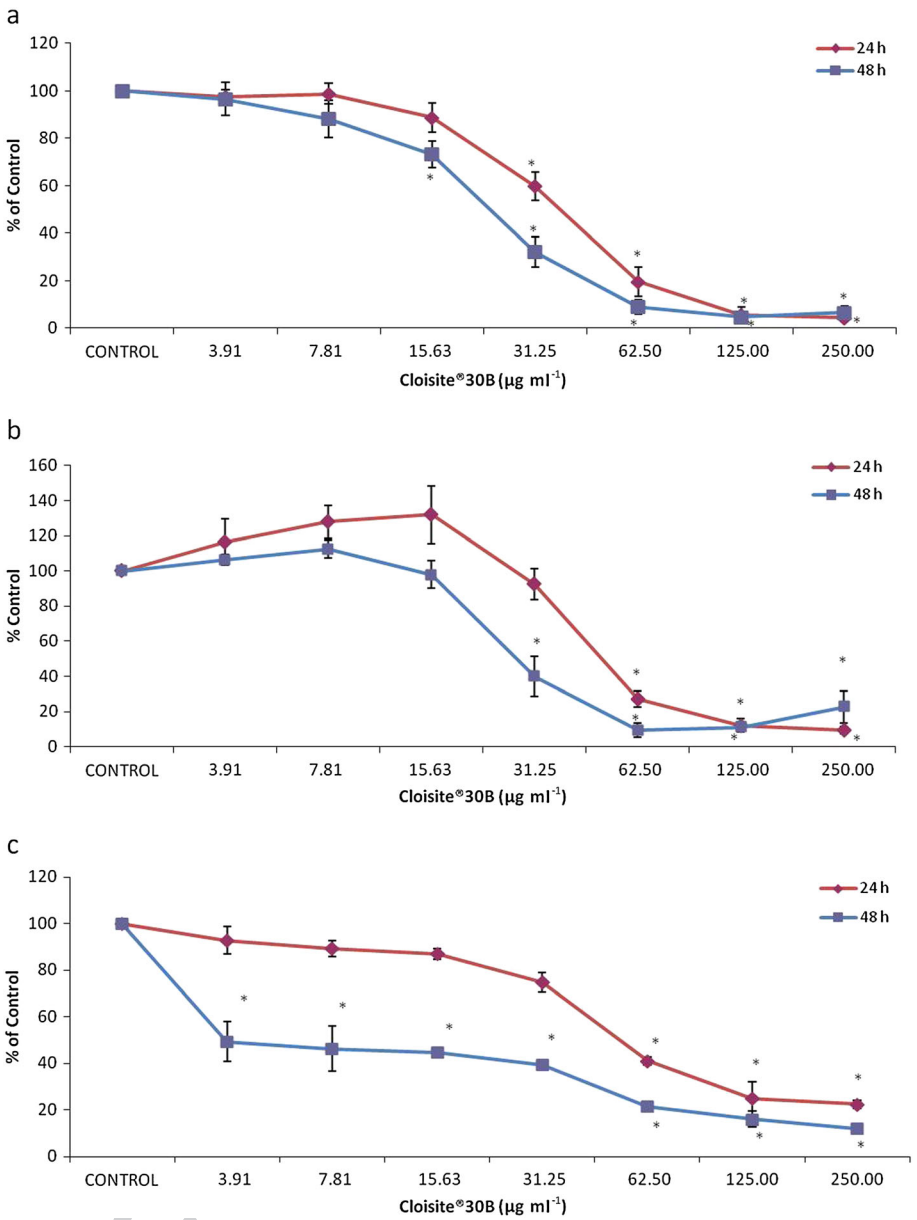

Figure 2. Protein content (a); neutral red uptake (b); and reduction of tetrazolium salt (c) of Caco-2 cells after 24 h and 48 h of exposure to 0–250 μg ml^{-1} Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.05$).

for 24 h and 40 ± 1 μg ml^{-1} for 48 h). Similarly, MTS reduction performed by cells exposed to Cloisite® 30B also decreased in a concentration- and time-dependent way, showing significant changes with respect to the control group from 62.50 μg ml^{-1} at 24 h of exposure, and from 3.91 μg ml^{-1} at 48 h (Fig. 2c). The EC_{50} values for the MTS assay also evidenced a greater effect in cells after a longer exposure time (79 ± 4 μg ml^{-1} for 24 h in comparison to 12 ± 1 μg ml^{-1} for 48 h); this difference was statistically significant.

Results of the Oxidative Stress Assays and Comet Assay

When Caco-2 cells were exposed to 10 and 20 μg ml^{-1} Cloisite® 30B, the ROS content was not altered; whereas at exposure to 40 μg ml^{-1} Cloisite® 30B it enhanced significantly with respect to the control group at both exposure times (Fig. 3a). Similarly, GSH content only suffered a significant decrease at 40 μg ml^{-1} Cloisite® 30B after 48 h (Fig. 3b). However, Caco-2 exposed to Cloisite® 30B did not induce any DNA damage at the concentrations assayed (Fig. 4).

Results of the Morphology Study

Light microscope observation. Caco-2 cultured cells exhibit morphological features characteristic of differentiated cells, with a tendency to form aggregates, making it difficult to obtain a homogeneous culture. A decrease in cell growing is observed when cells are treated with 20 μg ml^{-1} Cloisite® 30B. Meanwhile, after exposure to the highest concentration assayed, 40 μg ml^{-1} Cloisite® 30B, cell death is induced. When cells are observed under phase contrast microscopy, morphological changes are more evident from the concentration of 20 μg ml^{-1} Cloisite® 30B. Intense vacuolization in the cytoplasm is observed in cells exposed to 20 and 40 μg ml^{-1} Cloisite® 30B (Fig. 5b,e,f). Most of

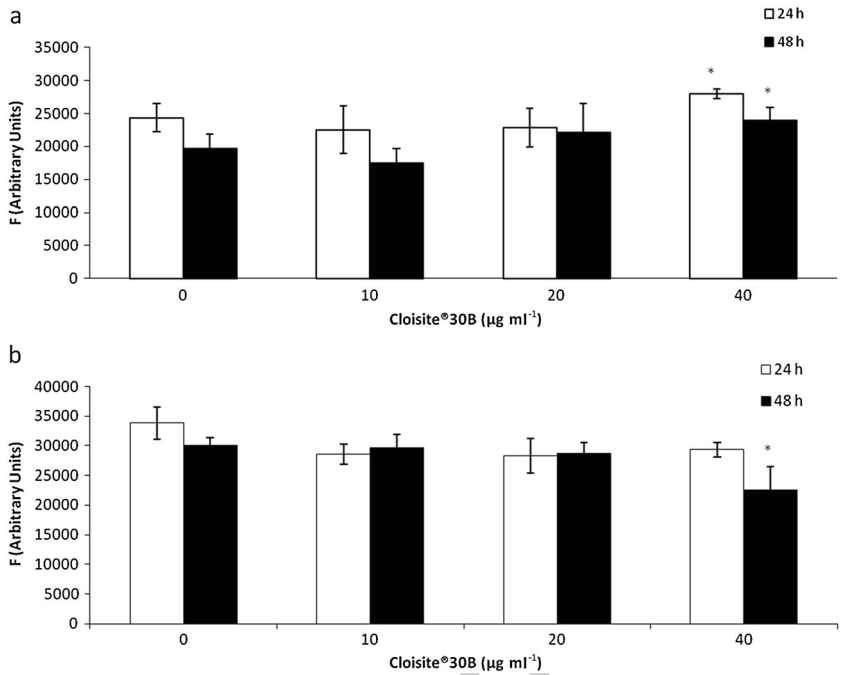

Figure 3. Reactive oxygen species content (a), and glutathione content (c) in Caco-2 cells after 24 and 48 h of exposure to 10, 20 or 40 µg ml^{-1} Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.05$).

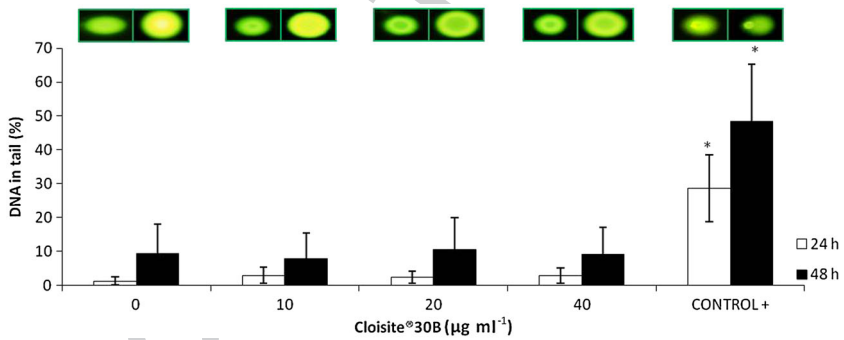

Figure 4. Comet assay results of Caco-2 cells after 24 and 48 h of exposure to 10, 20 or 40 µg ml^{-1} Cloisite® 30B. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.05$).

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the vacuoles correspond to lipid drops. Large euchromatic irregular nuclei are also observed (Fig. 5b,c). Moreover, as it has been already mentioned, cell death is induced and apoptotic cells are shown in the exposure to 40 µg ml^{-1} Cloisite® 30B (Fig. 5c).

Electron microscope observation. When unexposed Caco-2 cells after 24 h of culture are observed by electron microscopy, they show euchromatic nuclei (Fig. 6a) with regular surface and prominent nucleoli. Numerous ribosomes are observed free in the cytoplasm (Fig. 6b). Mitochondria with light matrix and visible crest (Fig. 6b), intermediate filaments (Fig. 6a) and lipid drops (Fig. 6a) are also shown in the cytoplasm. The endomembrane system, especially in the Golgi apparatus, is very well developed (Fig. 6b). When unexposed Caco-2 cells are observed after 48 h of culture, slight changes are observed in the nucleus, with a more irregular nuclear surface (Fig. 6c). In the cytoplasm, an increase in the lipid drop content is visible (Fig. 6c) as well as tonofilaments. Mitochondria show light matrix and scarce thin crest (Fig. 6d). The most remarkable ultrastructural feature after 24 and 48 h of exposure to the lowest concentration assayed, 10 µg ml^{-1} Cloisite® 30B, is nucleolar segregation (Fig. 6e), showing the fibrillar component completely separated from the granular one (Fig. 6e). In the cytoplasm, the increase in the number of lipid drops (Fig. 6f) and degradation in the mitochondrial crest are observed. The endomembrane systems, especially the dictyosomes (Fig. 6g) show dilated cisternae edges (Fig. 6g).

All the morphological alterations previously observed were more severe when Caco-2 cells were exposed to 20 µg ml^{-1} Cloisite® 30B during 24 and 48 h, with mitochondria exhibiting matrix and inner membrane degradation, and dilated dictyosomes (Fig. 7a). Moreover, morphological signs of cellular differentiation are observed, such as membrane specializations forming microvilli, development of endocytic vesicles and dilated cisternae from the rough endoplasmic reticulum (Fig. 7b). However, the most remarkable changes were observed when cells were exposed to the highest concentration, 40 µg ml^{-1} Cloisite® 30B (Fig. 7c–f). Heterophagosomes with laminar content and

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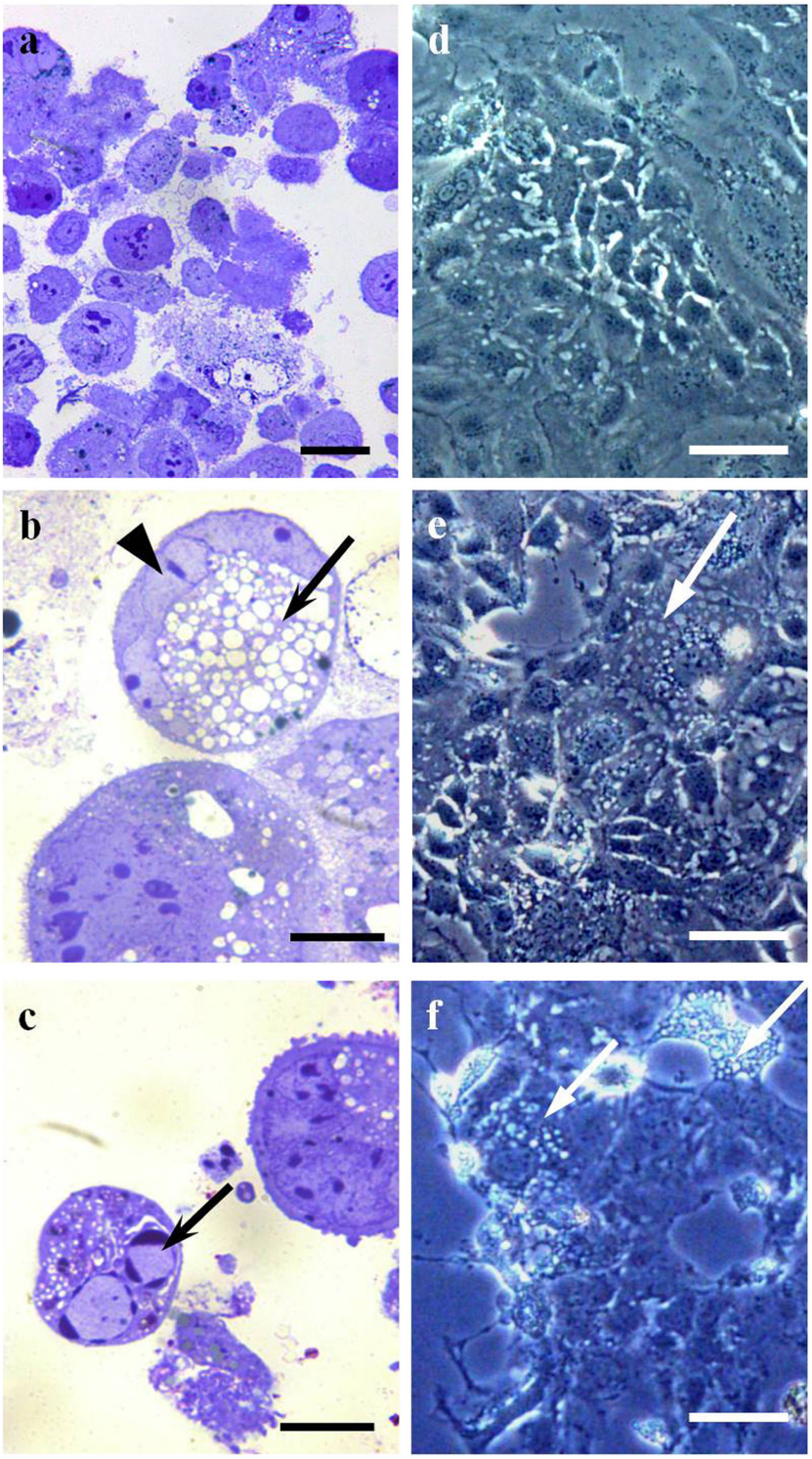

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Figure 5. Morphology of Caco-2 cells observed by bright-field microscopy (a–c) and phase contrast microscopy (d–f). Unexposed control culture (a,d). Cells exposed for 48 h to 20 μg ml^{-1} (e) or 40 μg ml^{-1} Cloisite® 30B (b.c.f). (a) Bar = 200 μm. (b–f) Bars = 100 μm. (b) Intense vacuolization in the cytoplasm (black arrow) and large euchromatic irregular nuclei (black arrowhead). (c) Apoptotic cells (black arrow). (e,f) Vacuolization in the cytoplasm (white arrow).

small vesicles with endocytic origin are visible in the cytoplasm. The laminar structure observed in the heterophagosome could correspond to agglomerated clay. Moreover, intense dilatation in the trans face of Golgi apparatus, autophagosome with mitochondrial origin, lobed nucleus sections and lipid drops are also observed (Fig. 7c). Scarce viable cells are distinguished in the culture showing nucleus with irregular surface (Fig. 7d), cytoplasm intensely vacuolated (Fig. 7d) as a result of mitochondrial degradation (Fig. 7e), and presence of lipid drops (Fig. 7e), secondary lysosomes (Fig. 7e), segregated nucleoli (Fig. 7e) and dilated cisternae in the Golgi apparatus and rough endoplasmic reticulum (Fig. 87).

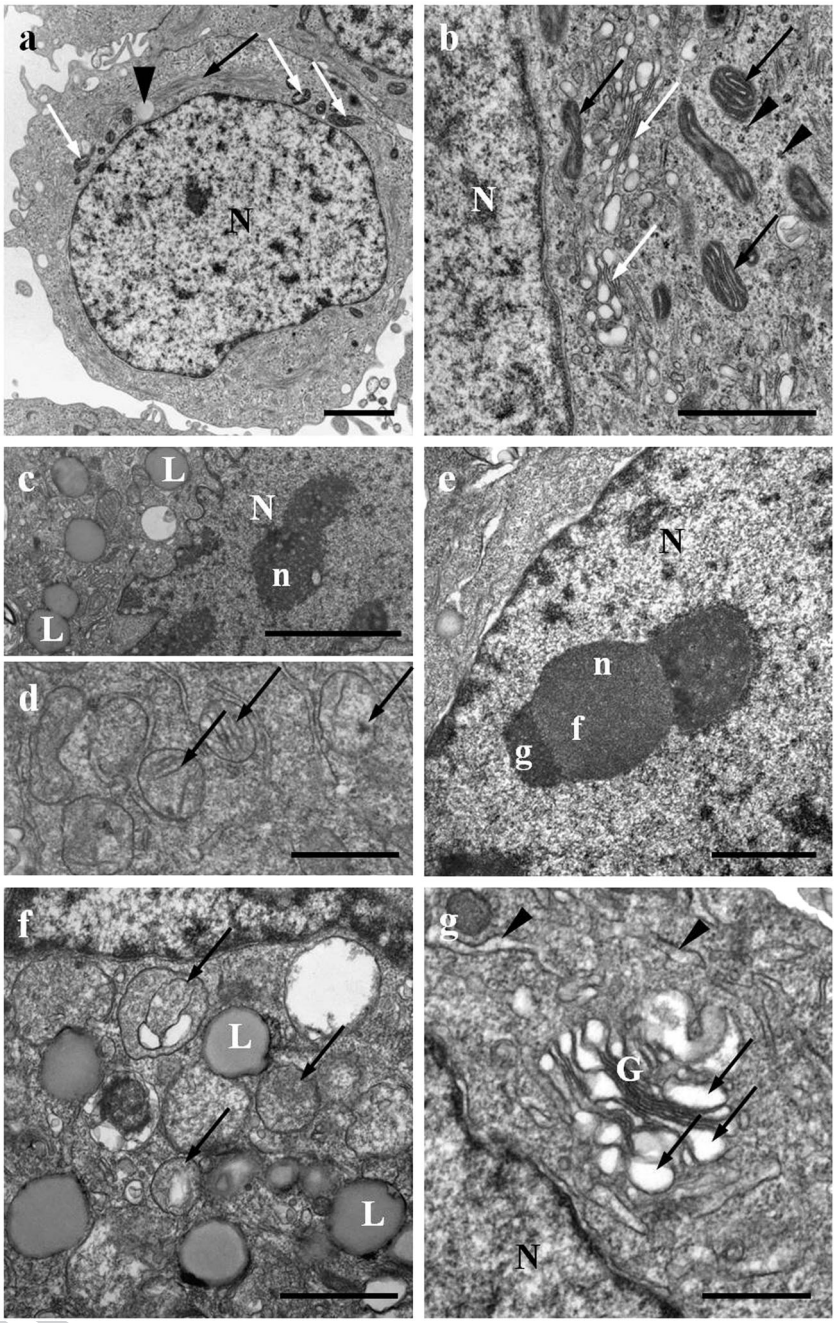

Figure 6. Ultrastructural features of unexposed Caco-2 cells and after exposure to Cloisite® 30B observed by electron microscopy. Unexposed control cells after 24 h of culture (a,b) and after 48 h (c,d), and Caco-2 cells exposed to 10 μg ml^{-1} Cloisite® 30B (e–g). (a) Unexposed cells showing euchromatic nuclei with regular surface (N), mitochondria (white arrow), intermediate filaments (black arrow) and lipid drops (black arrowhead). Bar = 2 μm. (b) Numerous polyribosomes in the cytoplasm (black arrowhead), mitochondria with light matrix and visible crest (black arrow), well developed Golgi apparatus (white arrow). Bar = 2 μm. (c) Unexposed Caco-2 cells after 48 h of culture show nuclei (N) with irregular surface, nucleolus (n) and lipid drops in the cytoplasm (L). Bar = 2 μm. (d) Mitochondria with light matrix and scarce thin crest (black arrow). Bar = 2 μm. (e) Exposed Caco-2 cells show nucleolar segregation (n), showing the fibrillar component (f) completely separated from the granular one (g). Bar = 1 μm. (f) Increase in the number of lipid drops (L) and degradation in the mitochondrial crest (black arrow) are observed in the cytoplasm. Bar = 1 μm. (g) Dictyosomes (G) show dilated cisternae edges (black arrow) and dilated cisternae from the rough endoplasmic reticulum (black arrowhead). Bar = 0.5 μm.

Discussion

Because of the growing use of clays, human exposure is inevitable and so an evaluation of their potential toxicity is necessary (Lordan *et al.*, 2011). In the present study, biochemical and morphological alterations of two different clays, the natural mineral sodium montmorillonite Cloisite® Na^+ and the same clay modified with a quaternary ammonium compound (Cloisite® 30B) were studied in the human cell line Caco-2. Results obtained showed no significant cytotoxic effects in Caco-2 cells exposed to the selected concentrations of Cloisite® Na^+ for 24 and 48 h; however, all the endpoints assayed for Cloisite® 30B appeared altered. In this sense, other authors have also studied the cytotoxic effects of different clays in permanent cell lines

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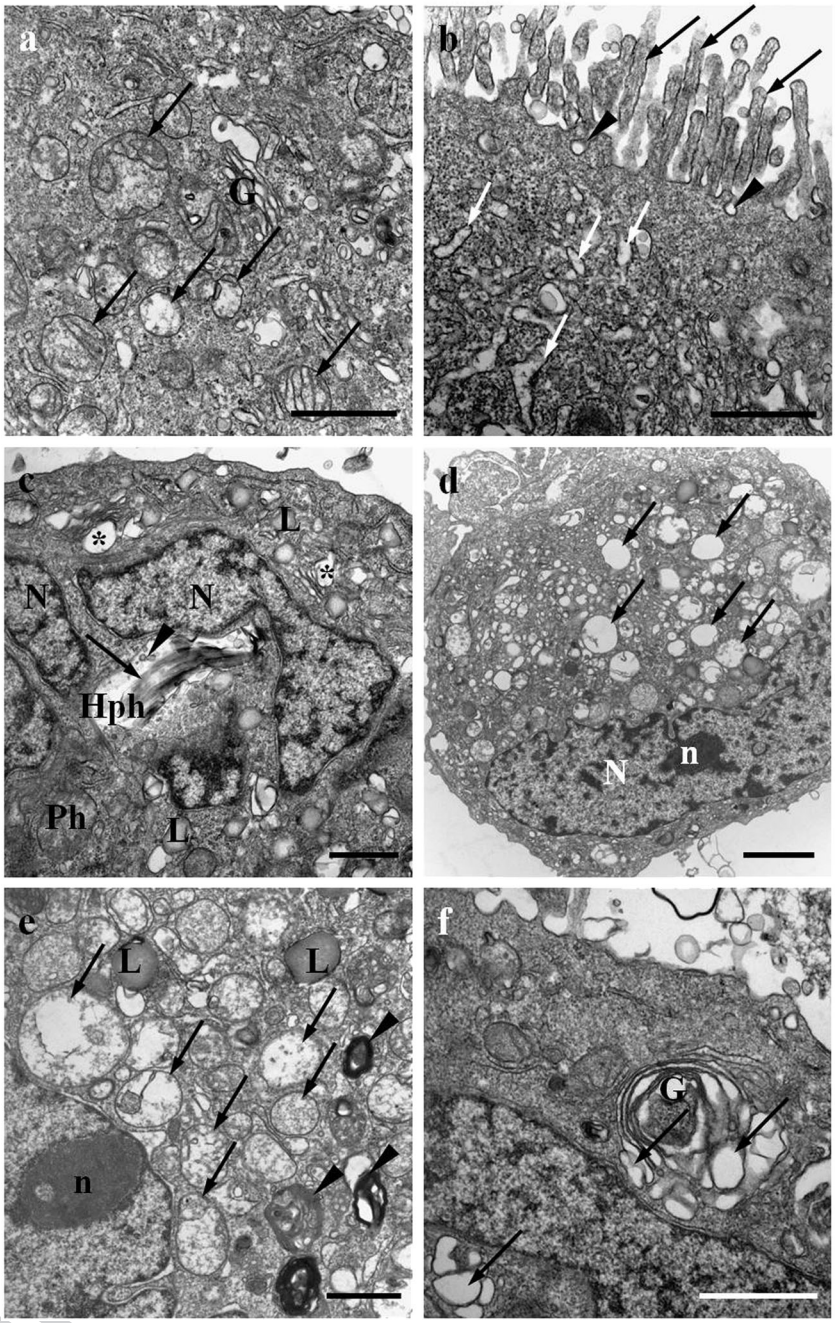

Figure 7. Ultrastructural changes of Caco-2 cells after exposure to Cloisite® 30B observed by electron microscopy. Caco-2 cells exposed to 20 μg ml^{-1} Cloisite® 30B (a,b) or 40 μg ml^{-1} Cloisite® 30B (c–f). (a) Cells with mitochondria exhibiting matrix and inner membrane degradation (black arrow), and dilated Golgi apparatus (G). Bar = 1 μm. (b) Differentiated cells show membrane specializations forming microvilli (black arrow), development of endocytic vesicles (black arrowhead) and dilated cisternae from the rough endoplasmic reticulum (white arrow). Bar = 2 μm. (c) Heterophagosome (Hph) with laminar content (arrow) and small vesicles with endocytic origin (arrowhead) are visible in the cytoplasm. Intense dilatation in the trans face of Golgi apparatus (asterisc), autophagosomes with mitochondrial origin (Ph), lobed nucleus sections (N) and lipid drops (L) are also observed. Bar = 2 μm. (d) Scarce viable cells are distinguished in Caco-2 culture exposed to 40 μg ml^{-1} Cloisite® 30B, showing nucleus with irregular surface (N), segregated nucleoli (n) and cytoplasm intensely vacuolated (black arrow). Bar = 1 μm. (e) Mitochondrial degradation (black arrow), and presence of lipid drops (L), secondary lysosomes (black arrowhead) and segregated nucleoli (n). Bar = 1 μm. (f) Dilated cisternae (black arrow) in the Golgi apparatus (G). Bar = 1 μm.

(Gao *et al.*, 2000; Geh *et al.*, 2006a; Li *et al.*, 2010). Murphy *et al.* (1993a) highlighted the importance of studying the effects of montmorillonite as this clay exhibited the most toxic effects in different cell lines, being the order of tested particles: montmorillonite bentonite = kaolin > > erionite.

Surface modification of clays has been commonly used to achieve a greater compatibility of the clay with polymer, and therefore reach higher exfoliation degrees, and in consequence better properties (Hetzer and De Kee, 2008). However, it has been suggested that the modification introduced in the

montmorillonite substantially changes its behavior in the experimental model. Actually, it changes the hydrophilic characteristics of the clays, and therefore their toxicity can be affected. In this sense, the oligo (styrene-co-acrylonitrile)-modified montmorillonite showed little cytotoxicity in mouse embryonic fibroblast (NIH 3T3) cells and human embryonic kidney 293 cells, and lower level of apoptosis was induced when cells were incubated with a high montmorillonite content (1 g l^{-1}) (Liu *et al*., 2011). However, a concentration-dependent response in HepG2 cells exposed to Cloisite® Na^+ and Cloisite® 93A was evident following 24 h of treatment with each of the clays, with a significant decrease in viable cells observed from 1 μg ml^{-1} (Lordan *et al*., 2011). In our study, the modification of the montmorillonite Cloisite® Na^+, to turn into Cloisite® 30B, produced an increase in the toxic effects observed in Caco-2 cells. Thus, for example after 48 h of exposure 62.5 μg ml^{-1} the percentage of viable cells measured with the MTS assay was 72% with Cloisite® Na^+ and only 21% with Cloisite® 30B.

In addition, the mechanism of montmorillonite to produce toxic effects is still unknown. The implication of oxidative stress in the toxic effects induced by Cloisite® 30B has been assayed in the present work, showing increases in the ROS content only when cells were exposed to the highest concentration tested (40 μg ml^{-1}) at both exposure times (24 and 48 h). The production of ROS could be related to the cell damage induced by Cloisite® 30B at this concentration that was also corroborated with the morphological study. Similarly, Lordan *et al*. (2011) reported that Cloisite® Na^+ induced intracellular ROS formation, which coincided with increased cell membrane damage, while ROS generation did not play a role in Cloisite 93A-induced cell death in human hepatoma cells (HepG2). By contrast, Sharma *et al*. (2010) found that Cloisite® Na^+ and Cloisite® 30B did not produce ROS in a cell-free test system at the concentrations assayed (0–226 μg ml^{-1}).

Furthermore, no genotoxicity was found in the present work for Cloisite® 30B in the comet assay on Caco-2 cells exposed to 10, 20 and 40 μg ml^{-1} for 24 and 48 h. However, higher concentrations of unfiltered and filtered Cloisite® 30B samples (113 and 170 μg ml^{-1}) were found to be genotoxic in a concentration-related manner in the same cell line (Sharma *et al*., 2010). Moreover, genotoxic effects of different clays (bentonite kaolinite, quartz and exfoliated silicate nanoclay) on several experimental models have been previously reported (Gao *et al*., 2000; Geh *et al*., 2006b; Li *et al*., 2010; Meibian *et al*., 2011).

Despite several morphological studies have been conducted on animals and cell lines exposed to montmorillonite, to our knowledge, this is the first work reporting ultrastructural changes in the intestinal cell line Caco-2 exposed to two different types of clays. The most remarkable morphological changes observed in the present work were degraded mitochondria, increase in the presence of lipid drops, dilated cisternae edge in the Golgi apparatus and nucleolar segregation. The nucleolar segregation found in the present study was previously described in HeLa cells treated with 2 μg ml^{-1} actinomycin D for 5 h, in which a segregated nucleolus with the fibrillar component forming a cap-like structure in the nucleolar periphery, separated from the granular component was observed (Rendón *et al*., 1992). Actinomycin D is known to inhibit the synthesis of proteins by inhibiting the rRNA synthesis (Rendón *et al*., 1992); therefore, considering the similarities in ultrastructural findings observed in our work, they could be due to inhibition in the synthesis of protein caused by montmorillonite; although it has not been confirmed. Consequently, to this inhibition, impairment in the secretory functions has been observed in the dilatation of the Golgi apparatus, probably due to the accumulation of glycoprotein. Ratcliffe *et al*. (1985) reported accumulation of glycoprotein in the Golgi apparatus in chondrocytes treated with monensin, as it interferes with intracellular translocation of secretory proteins in many different cell types inhibiting transport within the Golgi complex.

Moreover, *in vivo* morphological studies have reported that a non-modified montmorillonite used to pharmaceutical purposes was non-toxic to Wistar rats (Lee *et al*., 2005). Additionally, copper bearing montmorillonite dietary exposed to pigs and broilers may improve small intestinal mucosal morphology (Xu *et al*., 2003; Xia *et al*., 2005). However, *in vitro* studies have evidenced cell damage induced by clays. In this sense, Murphy *et al*. (1993a) performed a similar assay on HUVEC, N1E-115 and ROC1 cells exposed to 100 μg ml^{-1} of aluminum silicate-containing montmorillonites showed morphological alterations with cell death, as well as an increase in the release of fatty acids and decrease of cell viability measured by Trypan Blue exclusion after 24 h of exposure. This research group also reported cell lyses in neuronal cultures within 60 min following exposure to aluminum silicate-containing bentonite and montmorillonite (Murphy *et al*., 1993b). Moreover, Banin and Meiri (1990) reported clear morphological signs of cell deterioration in murine neuroblastoma cells exposed to montmorillonite. In addition, bentonite samples induced necrotic as well as apoptotic cell death in human lung fibroblasts, although in the morphological study, no cell organelles were affected (nucleus, mitochondria or endoplasmic reticulum) after 24 h of exposure to 10 μg cm^{-2} of bentonite (Geh *et al*., 2006a). In the present work, the laminar structure observed in the heterophagosome could correspond to agglomerated clay. In this sense, Woodworth *et al*. (1982) also observed by scanning electron microscope and phase contrast microscopy that tracheal epithelial cells exposed to montmorillonite phagocytized the particle. Moreover, they reported that cells containing intracellular particles demonstrated retraction of lamellopoidal extensions, surface blebbing and a changed morphology from flattened to round (Woodworth *et al*., 1982). In addition, Geh *et al*. (2006a) reported that activated bentonite particles are better taken up by human lung cells than untreated (native) bentonite particles. Concerning intestinal cells, the uptake of compounds in the gastrointestinal tract depends on diffusion and accessibility through mucus, initial contact with the gut epithelium and various uptake and translocation processes (Bouwmeester *et al*., 2009). Once they pass the gastrointestinal epithelium and end up in the blood circulation, they are distributed around the body (Nemmar *et al*., 2002). In the present study, the concentrations assayed are lower than those used in the manufacture of food contact materials and consequently human contact with toxic concentrations is feasible considering the worst case scenario of exposure. Considering that polymer nanocomposites used as food contact materials contain 4% of clay, a rigid packaging of 20 g (a small bottle) would contain 0.8 g of clay. Our results have shown that 250 μg ml^{-1} in the well of a cell culture plate, which is 50 μg Cloisite 30B, induce a high cytotoxicity. In addition, EFSA (2011) considers that in the absence of exposure data (as in our case), and where it is not possible to determine the nanoform in the food matrix, it should be assumed that all added nanomaterial is present, ingested and absorbed in the nanoform. Therefore, if the clay contained in the polymer reaches the consumer, the

potential of toxic effects is not negligible. Our results indicate that a case by case toxicological evaluation of clays is needed as modifiers change their toxicity profile. Moreover, further research is required to clarify the uptake of the clay into the cell as well as its kinetics for a better understanding of its toxic mechanism.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study. Moreover, we thank the Cell Culture Service and Microscopy Service of CITIUS (University of Seville) and Remedios García Navarro for providing technical assistance.

Conflict of Interest

The authors did not report any conflict of interest.

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Q8	AUTHOR: Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain)—where in Spain for these manufacturers?	
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Q10	AUTHOR: Termogravimetric analysis showed a large loss of mass for the Cloisite® 30B—OK now?	
Q11	AUTHOR: NuAire®, Spain—pls add manufacturer/supplier and place in Spain.	
Q12	AUTHOR: For this reason the absorbance of clay solutions (1000 μg ml^{-1} and serial half dilutions) were measured at 0, 24 and 48 h.—OK now?	
Q13	AUTHOR: Dr. Hielscher, Germany—is this a manufacturer or is it a kind gift from someone? Please add place in Germany.	
Q14	AUTHOR: Tecan, Austria—where in Austria?	

Query No.	Query	Remark
Q15	AUTHOR: being this endpoint a good marker of the damage induced in this organelle.—sense?	
Q16	AUTHOR: and the concentrations used here for Cloisite® 30B were 0, 10, 20 and 40 μg ml^{-1}.—OK now?	
Q17	AUTHOR: Molecular Probes, Invitrogen—pls add address. Am assuming this is 1 manufacturer and not 2?	
Q18	AUTHOR: Perceptive Instruments, UK—where in UK (add town/city and county)?	
Q19	AUTHOR: Leitz (Aristoplan) light microscope—please add address.	
Q20	AUTHOR: Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope. The cell growth and development of morphology damage was checked using a Leica DMIL inverted microscope by phase contrast and photographed using a Leica DC 100 camera.—please add addresses.	
Q21	AUTHOR: copper bearing montmorillonite dietary exposed to pigs and broilers—sense? Is there something missing here.	
Q22	AUTHOR: 'research group'—OK now?	
Q23	AUTHOR: Reference "Heikkilä et al. (2010)" is not cited in the text. Please indicate where it should be cited; or delete from the reference list.	

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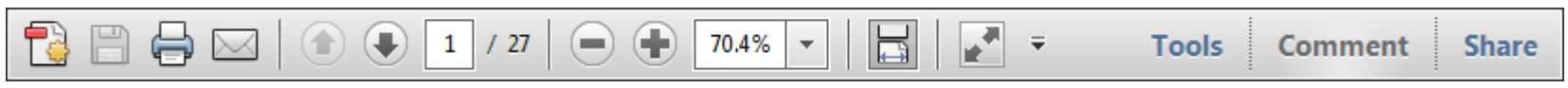
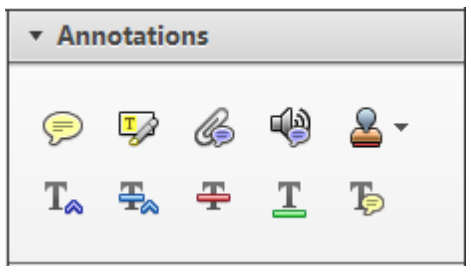
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

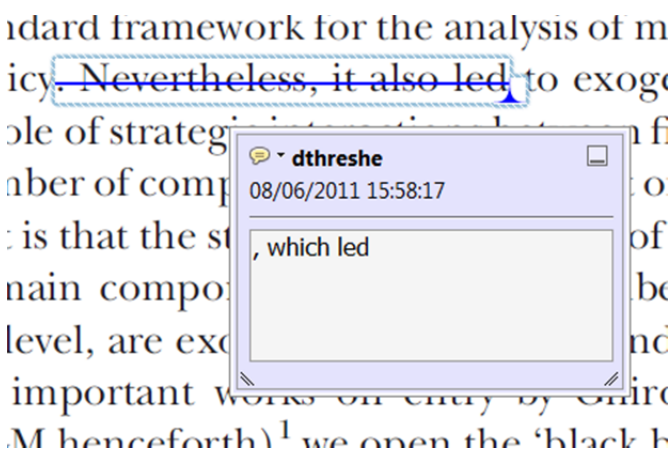


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

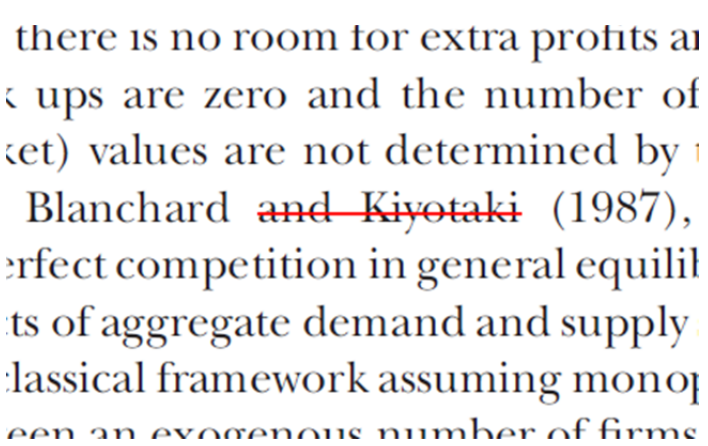


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

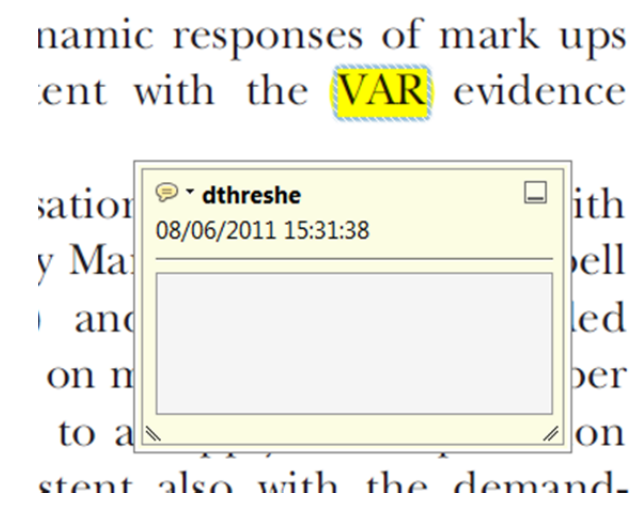


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

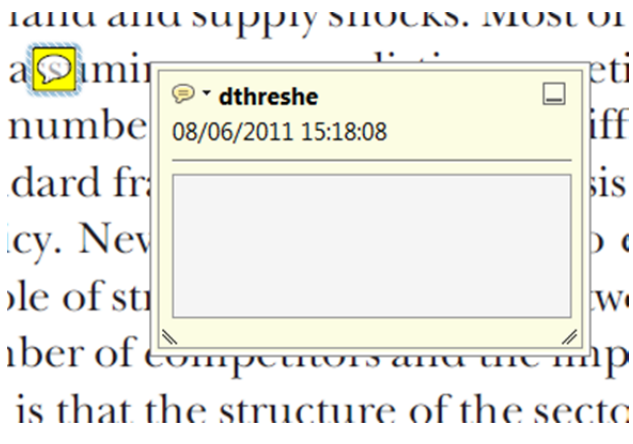


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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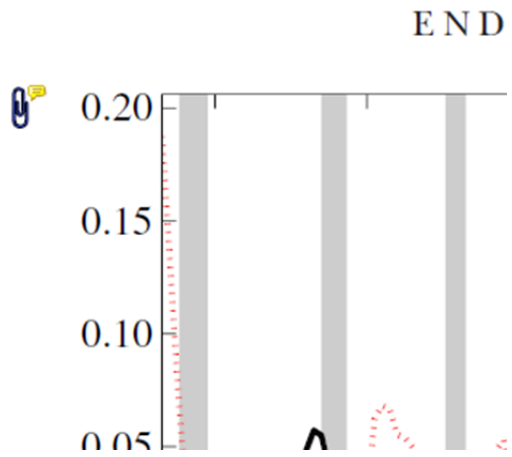
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

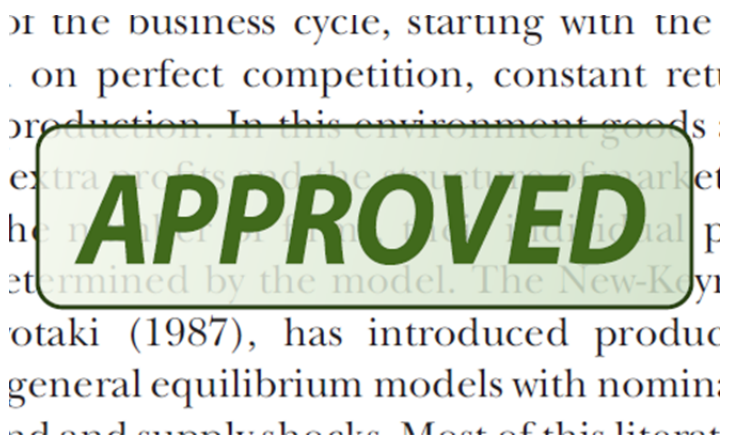


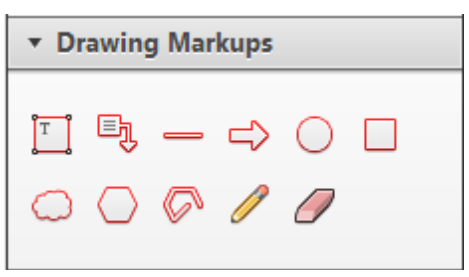
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



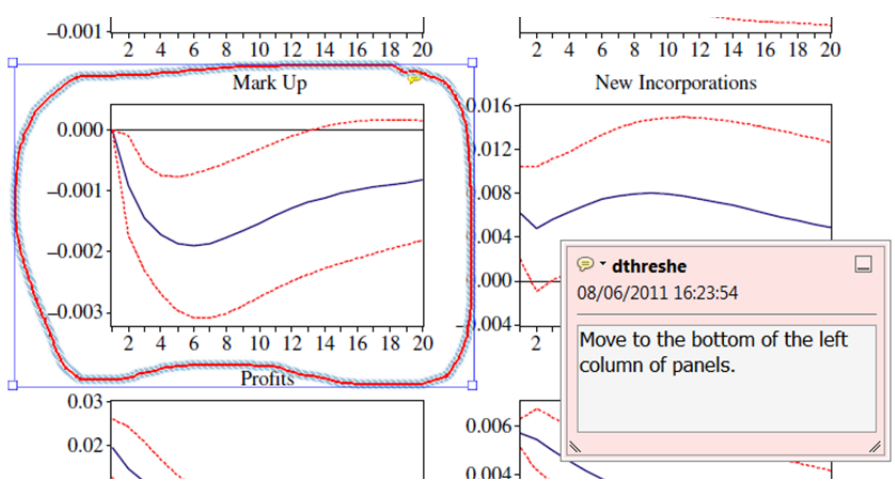


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

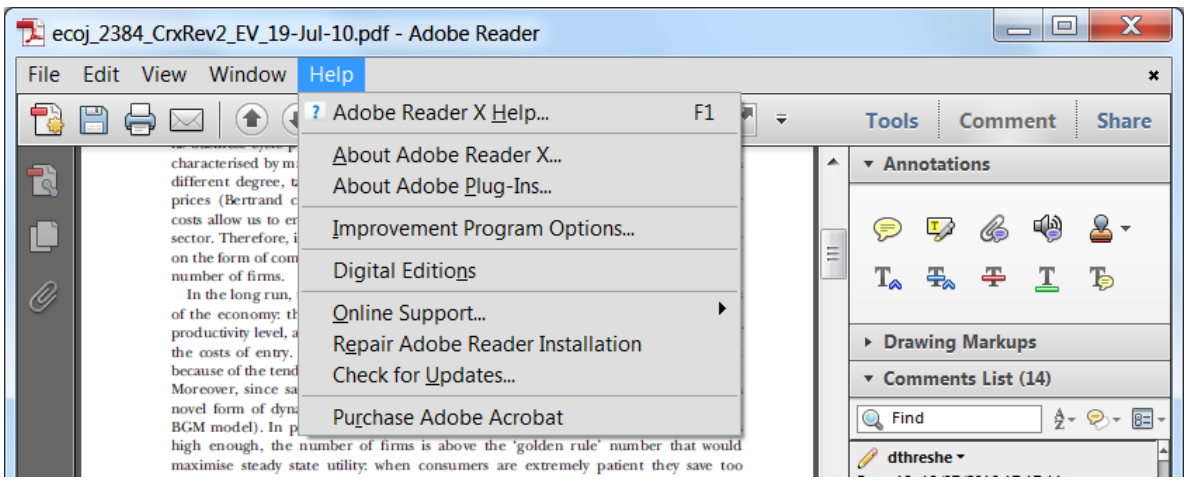
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



CAPÍTULO 7 / CHAPTER 7

Houtman J, **Maisanaba S,** Puerto M, Gutiérrez-Praena D, Jordá M, Aucejo S, Jos A

TOXICITY ASSESSMENT OF ORGANOMODIFIED CLAYS USED IN FOOD CONTACT MATERIALS ON HUMAN TARGET CELL LINES

Aplied Clay Science 90, 150-158, ***2014***

Elsevier Editorial System(tm) for Applied Clay Science
Manuscript Draft

Manuscript Number: CLAY5406R2

Title: Toxicity assessment of organomodified clays used in food contact materials on human target cell lines

Article Type: Research Paper

Keywords: Clay; montmorillonite; cytotoxicity; cell lines; DNA damage

Corresponding Author: Mrs. María Puerto,

Corresponding Author's Institution: University of Seville

First Author: Judith Houtman

Order of Authors: Judith Houtman; Sara Maisanaba ; María Puerto; Daniel Gutiérrez-Praena ; María Jordá; Susana Aucejo; Angeles Jos

Abstract: Nowadays, the incorporation of organomodified clays based on montmorillonite into polymers intended for packaging industry is a reality. The final result is a polymer nanocomposite with enhanced barrier properties. Different organomodified clays are already commercially available and others new ones are being developed, however little is known about their safety.
In the present work, the cytotoxic effects (a tetrazolium salt reduction and protein content) of three organomodified clays, Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified clays developed by the Packaging, Transport, & Logistics Research Institute, were evaluated in Caco-2 and HepG2 cells after 24 and 48h of exposure. Our results showed that only Clay 2 induced toxic effects in both cell lines. The mean effective concentration was calculated for each case, showing Caco-2 to be more sensitive than HepG2. Moreover, in order to elucidate the toxicity mechanisms of Clay 2, different mechanistic biomarkers were investigated. Interleukin leakage and generation of intracellular reactive oxygen species was not observed, whereas glutathione content decreased in HepG2. DNA damage (comet assay) was induced in both cell lines at the highest concentration tested. Overall, results show that the type of clay, the concentrations range and the type of cell line play an important role in the toxicity observed.

Cover Letter, for Editor only

María Puerto
Area of Toxicology. Faculty of Pharmacy. University of Seville
C/Profesor García González 2, 41012 Seville, Spain
Tel.: +34-954556762; fax: 34-954556422
E-mail address: mariapuerto@us.es

September, 13 2013

Dear Editor,

Thank you very much for giving us the opportunity again to submit a revised version of our manuscript entitled “Toxicity assessment of organomodified clays used in food contact materials on human target cell lines” for its publication in “**Applied Clay Science**”.

The authors of the article were: Judith Houtman, Sara Maisanaba, María Puerto, Daniel Gutiérrez-Praena, María Jordá, Susana Aucejo, Ángeles Jos.

We thank the review performed. We hope they fulfill reviewer’s suggestions.

Looking forward to receiving a positive answer from you.

Sincerely,

María Puerto

Toxicity assessment of organomodified clays used in food contact materials on human target cell lines

Houtman J[1], Maisanaba S[2], Puerto M[2*], Gutiérrez-Praena D[2], Jordá M[3], Aucejo S[3], Jos A[1]

[1]Wageningen University and Research Center. Wageningen. The Netherlands.

[2]Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[3]Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

*Corresponding author:

María Puerto

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: mariapuerto@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Nowadays, the incorporation of organomodified clays based on montmorillonite into polymers intended for packaging industry is a reality. The final result is a polymer nanocomposite with enhanced barrier properties. Different organomodified clays are already commercially available and others new ones are being developed, however little is known about their safety.

In the present work, the cytotoxic effects (a tetrazolium salt reduction and protein content) of three organomodified clays, Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified clays developed by the Packaging, Transport, & Logistics Research Institute, were evaluated in Caco-2 and HepG2 cells after 24 and 48h of exposure. Our results showed that only Clay 2 induced toxic effects in both cell lines. The mean effective concentration was calculated for each case, showing Caco-2 to be more sensitive than HepG2. Moreover, in order to elucidate the toxicity mechanisms of Clay 2, different mechanistic biomarkers were investigated. Interleukin leakage and generation of intracellular reactive oxygen species was not observed, whereas glutathione content decreased in HepG2. DNA damage (comet assay) was induced in both cell lines at the highest concentration tested. Overall, results show that the type of clay, the concentrations range and the type of cell line play an important role in the toxicity observed.

Keywords: clay, montmorillonite, cytotoxicity, cell lines, DNA damage

1. Introduction

Over the last decades, the use of polymers as food packaging materials has increased enormously due to their advantages over other traditional materials (Jordan et al., 2005; Silvestre et al., 2011). Latest innovations include the use of "improved" food contact materials, this is the addition of different reinforcements, for example nanoparticles, to the polymeric matrix in order to enhance the functional properties of packaging materials, and thus improve the shelf life of food and beverage products. The resulting nanocomposites are a multi-phase material in which the majority of the dispersed phase components have one or more dimensions of the order of 100 nm or less (SCENIHR, 2007).

Smectite clays, such as montmorillonite (MMT), belong to the structural family called 2:1 phyllosilicates, which present a structure composed by two tetrahedral layers formed by Si and O atoms, fused with an octahedral layer with aluminum and magnesium atoms bonded to oxygen and hydroxyl groups (Jordá-Beneyto et al., 2008). They are one of the main choices for designing polymer nanocomposites due to their low cost and rich intercalation chemistry allowing them to be chemically modified (organoclays) and to improve the compatibility with the polymer matrix (Bitinis et al., 2011). Once the final clay nanocomposites are ready, the resulting material presents a specific disposition of the clays, such as platelets, giving a tortuous path to the gas permeant, forcing it to travel a longer path to diffuse through the film (Nielsen, 1967). Several authors reviewed the advantages, and in few cases the limitations, of the layered nanocomposites. Great improvements in thermal, mechanical and barrier (permeability) properties are presented, as well as strength, stiffness, dimensional stability, and heat resistance (De Azeredo, 2009; Duncan et al., 2011; Hatzigrigoriou et al., 2011; Hetzer et al., 2008). Moreover, they also offer good barrier properties under different packing, handling, shipping, and

storage conditions (Avella et al., 2005; Brody, 2006; Ray et al., 2006; Timoty and Duncan 2011; Volpe, 2005), therefore they could improve the quality and safety of packaged food (Lagaron,2006).

Although there is a lot of evidence for the good technological performance of nanocomposites, safety issues are also of importance. Available data on clay's toxicity is still scarce, but different authors have already described toxic effects induced by montmorillonite and organoclays (~~Gutiérrez-Praena et al., 2013;~~ Lordan et al., 2011; Maisanaba et al., 2013a,c; Sharma et al., 2010). Moreover, the modifier used to synthesize the organoclay has a role in the toxicity observed (Maisanaba et al., 2013a).

In the case of organoclays, ~~T~~the oral pathway is the most important entrance route for the consumers, as they are exposed to the possible migrants from packaging nanocomposites to the food products, and they should know the possible effects of the ingestion of these nanosubstances to the gastrointestinal tract (Silvestre et al., 2011). When the nanoparticles reach the blood circulation, the liver is among the main organs for distribution (SCENIHR, 2009), so for clays imbued in the nanocomposite material this possibility cannot be discarded.

Taking into account that the production of nanocomposites is going to increase in the near future, the human exposure will also increase. Therefore, potential toxic effects should be investigated in order to guarantee the safety of the food products.

The aim of this study is to evaluate the toxicity of three modified montmorillonite clays used in the production of nanocomposites with applications as food contact materials in two target human cell lines of the digestive system, Caco-2 and HepG2 from intestinal and hepatocellular origin,

respectively. For this issue, basal cytotoxicity biomarkers and mechanistic biomarkers of inflammation, oxidative stress and genotoxicity have been investigated with Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified organoclays developed by the Technological Institute of Packaging, Transport and Logistics (ITENE).

2. Materials and Methods

2.1. Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Clays

Clay1 and Clay2 are two novel microsized clays that have been developed and characterized by Thermogravimetric Analysis (TGA) and Fourier Transform InfraRed (FTIR), as described in Maisanaba et al, (2013b) and Jordá-Beneyto et al., (2008, 2013).Clay 1 contains as modifier quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) and Clay 2 contains HDTA and acetylcholine chloride (ACO). Both clays are obtained by cation exchange reaction from Cloisite®Na^+ (Southern Clay Products, INC.). This raw clay has a typical dry particle size less than 25µm (d50). Cloisite®20A was also obtained from Southern Clay Products, INC. (modifier: dimethyl, dehydrogenated tallow, quaternary ammonium, concentration: 95 meq/100g clay). This modified clay has a typical dry particle size less than 10µm (d50) and has been characterized by Thermogravimetric Analysis (TGA) and Fourier Transform InfraRed (FTIR).

TGA analysis of Cloisite®20A and Cloisite®Na^+ (the original non-modified clay included as control) were performed on a Q5000IR thermobalance (TA Instruments) by heating the samples

from room temperature up to 900 °C with heating rate of 10 °C/min, in nitrogen atmosphere. Approximately 7 mg of each finely ground sample was heated in a platinum crucible.

FTIR Spectra were obtained on an Equinox 55 spectrometer (Bruker), coupled to a microscope modulus with ATR objetive (Hiperion, Bruker). This technique was used to characterize both clays Cloisite®Na^+ and Cloisite®20A. For each sample 128 scans were recorded with a resolution of 4 cm^{-1}.

2.3. Model systems

Caco-2 cell line derives from a human colon carcinoma (ATCC® HTB-37) and HepG2 is a human hepatocellular carcinoma epithelial cell line (ATCC® HB-8065). Both of them were obtained from the American Type Culture Collection. Caco-2 cell line was maintained in Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 µg/mL gentamicine, 2 mM L-glutamine, and 1 mM pyruvate. HepG2 cell line was maintained in EMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown near confluence in 75-cm^2 plastic flasks at 37ºC in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by the Trypan Blue exclusion test. The cells were used at passages between 10 and 21. Both cell lines were plated at a density of 7.5 x 10^5 cells/mL to perform all experiments.

2.4. Clays test solutions

Test concentrations of clays were determined individually in previous experiments in order to avoid interference with the method of measurement. With this purpose the absorbance of clay solutions (1000 µg/mL and serial ½ dilutions) were measured at 0, 24 and 48h. The

concentrations selected were the highest ones that did not show statistical differences versus the control. Thus, the maximum concentrations were 8 µg/mL for Clay 1, 125 µg/mL for Clay 2, and 62.5 µg/mL for Cloisite®20A. Test solutions were prepared in serum-free medium. An ultrasonic tip (Dr. Hielscher, Germany) at an amplitude of 40% for a total time of 30 s was employed to disperse the test concentrations.

2.5. Cytotoxicity assays

From the initial solutions, serial dilutions in medium without serum were prepared. Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37ºC for 24 and 48 hours. The basal cytotoxicity endpoints assayed were tetrazolium salt reduction (MTS) and protein content (PC).

MTS reduction is carried out by dehydrogenases, enzymes present in mitochondria, making this endpoint a good marker for the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium salt) added to the medium is reduced by the enzymes to a colored formazan product soluble in culture medium and is measured by a spectrophotometer at 490 nm after 2 hours of incubation in the dark.

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage with independence of the toxic mechanism involved (Pichardo et al. 2007). PC was quantified *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the total cell number present in the wells. The culture medium was replaced by

200 µL NaOH and after 2 h of incubation at 37ºC, 180 µL were replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm in a microplate spectrophotometer (Tecan Infinite M200, Austria).

2.6. Oxidative stress assays

Considering that only Clay 2 showed remarkable cytotoxic effects, this clay was chosen to perform mechanistic studies. For this purpose low cytotoxic concentrations were selected: 0, 8.5, 17 and 34 µg/mL for Caco-2 cells and 22, 44 and 88 µg/mL for HepG2 cultures. These concentrations correspond to the mean effective concentration (EC_{50}) value obtained for the most sensitive cytotoxicity endpoint at 24h along with the fractions $EC_{50}/2$ and $EC_{50}/4$. After replacing the previous medium, the exposure solutions were added to the cells, and incubated at 37ºC for 24 and 48 hours. Culture medium without clay was used as control group. The oxidative stress endpoints measured were reactive oxygen species (ROS) content and glutathione (GSH) levels.

The production of ROS was assessed in 96 well microplates using the dichlorofluorescein (DCF) assay. The probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Invitrogen) readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent compound (DCFH), which is rapidly oxidized in the presence of ROS to the highly fluorescent DCF. Specifically, cells were incubated with 200 µl 20 µM DCFH-DA in culture medium at 37ºC for 30 min, and then washed with phosphate buffered saline (PBS) and resuspended in 200 µl of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as fluorescence arbitrary units (Puerto et al. 2010).

GSH content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos et al. 2009). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme GST. After the cell exposure to the clay, medium was discarded and cells were incubated at 37°C for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the excitation/emission wavelengths of 380/460 nm. Results were expressed as arbitrary units.

2.7. Interleukin-6 leakage

For this assay, the culture medium of the cells after 24 and 48h exposure to Clay 2 was used. Manufacturer instructions from the kit (EH2IL6, Thermo Scientific, USA) were followed.

2.8. Comet assay

The comet assay was performed to detect DNA strand breaks. The cells lines were seeded into 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37ºC in 5% CO_2 to attach to the plates. Approximately 3.5×10^5 Caco-2 or HepG2 cells were exposed to different concentrations of Clay 2: 0, 8.5, 17, 34 µg/ml and 0, 22, 44 an 88 µg/ml, respectively, for 24 and 48 h.

In order to monitor the ongoing process of the assay, a negative control (cells treated with culture medium) and a positive control (cells treated with a solution of 100 µM H_2O_2) were included. After treatments cells were washed and detached in PBS. The comet assay was applied as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, cells were resuspended in PBS at a concentration of $2.5x10^6$ cells/mL. This suspension was mixed with 1% low melting point agarose and placed on a microscope slide (12 gels per slide). Once the gels

became solid, the slides were dipped into lysis solution at 4ºC. All nucleotides were denatured in a high-pH buffer. Electrophoresis was carried out at approximately 25 V (300 mA, ~ 1V/cm),and the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold nucleic acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20× objective) coupled via a CCD camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei (≥100) per experimental point were analyzed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK). The results from four independent experiments are expressed as % of tail DNA.

2.9. Calculations and statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration (n=3). Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out with GraphPad InStat3 software (La Jolla, CA, USA) using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. This test compares the response of the different exposure concentrations versus the controls. Previous to ANOVA, normality was checked with Kolmogorov–Smirnov test (p>0.05) and variance homogeneity among groups assessed with Bartlett's homocedasticity test (p>0.05). Differences among groups were considered significant at~~from~~ ~~P~~p<0.05.

EC_{50} values (mean effective concentration, concentration that modified each biomarker by 50%, positive or negative, in comparison with appropriate untreated controls) were derived by linear regression in the concentration-response curves.

3. Results

3.1. Characterization of Cloisite®20A

TGA results of both clays (Cloisite®Na^+ as control and Cloisite®20A) are presented in Figure 1. Loss weight percent (%), and derivative weight (%/°C) are presented versus temperature. It can be observed that only the sample Cloisite®20A has a big loss step in the range between 200 and 500 ºC, indicating that an organic compound is ~~being released in this step~~de~~s~~composed. The percentage of organic modifier in Cloisite®20A is 24.87 % w/w, as calculated in the TGA curves. Cloisite®Na^+ hardly presents any weight change in this range of temperature.

The presence of modifiers in the clays was determined by FTIR. The ATR spectra are shown in Figure 2. Characteristic Si-O stretching vibration band of the unmodified montmorillonite (cloisite Na+) occurs at ~~-~~1030 cm^{-1}, whereas in the spectra of the corresponding organoclay (cloisite20A) the band appears slightly shifted (1000 cm^{-1})(Madejova, 2003) ~~It can be observed the presence of a peak under 1200 cm^{-1} which corresponds to Si-O absorptions and OH bands that are typical of clay minerals~~. Inner hydroxyl groups, lying between the tetrahedral and octahedral sheets, gives the absorption near 3620 cm^{-1}, as it can be seen in both spectra in Figure 2. The C-H stretching bands of alkylammnium cations occur in the range of 3020-2800 cm^{-1}. The C-H stretching ~~Two strong~~ bands at~~near~~ 2930 cm^{-1} and at 2850 cm^{-1} are present in spectra of Cloisite®20A, which is a organo-modified clay. A characteristic C-H bending vibration of the $(CH3)4N^+$ cation at 1487 cm^{-1} is seen in the spectrum of the modified clay, which corresponds with the alkylammonium modifier in this clay. The spectrum of Cloisite®Na^+ does not present these peaks, showing that in this clay there is no presence of any organic modifier.

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3.2. Cytotoxicity assays

MTS reduction performed by Caco-2 and HepG2 cells exposed to Clay 1 remained unaltered with respect to the control cells after 24 and 48 h (Fig. 3a & 3b). In respect to PC, Caco-2 cell line

showed no alterations at 24 h, whereas at 48 h only experienced a significant (-p<0.05)- decrease respect to the control at 8 µg/mL (Fig. 3c). In the case of HepG2 cells, PC did not show significant changes with respect to the control after both exposure times, except at the highest concentration assayed in both cases (Fig. 3d). ~~* p<0.05 significantly different from control.~~

On the other hand, the MTS assay with Clay 2 presented a significant (-p<0.05) -decrease from 30 µg/mL at both exposure times in Caco-2 cell line (Fig. 4a). Similarly, MTS reduction in HepG2 cells presented a decrease from the concentration of 62.5 µg/mL after both exposure times, with significant differences at 48h (Fig. 4b). Moreover, Caco-2 cells showed a concentration and time-dependent decrease in PC, with significant (p<0.05) -reductions in cell viability from10 µg/mL onwards in cells exposed for 24 h and from 5µg/mL onwards after 48 h of exposure (Fig. 4c). Concerning to HepG2 cells, significant (p<0.05) changes appeared at the highest concentration assayed after 24 h, and from 62.5 µg/mL after 48 h (Fig. 4d). ~~* p<0.05 significantly different from control.~~

Regarding to Cloisite®20A, both cytotoxicity biomarkers assayed in Caco-2 and HepG2 cell lines, MTS (Fig. 5a & 5b) and PC (Fig. 5c & 5d), remained unaltered at any concentration assayed in respect to control cells after 24 and 48 h.

EC_{50} values could be obtained for Clay 2 only, showing a higher toxicity at the longest exposures (Table 1). Moreover, these data indicate that, in relation to cytotoxicity, Caco-2 cells have a higher sensitivity than HepG2 cells, as EC_{50} values are lower.

3.2. Oxidative stress assays

When Caco-2 or HepG2 cells were exposed to Clay 2 during 24 and 48 h, no significant alteration on ROS was observed at any of the exposure concentrations in comparison to the control group (Fig. 6a & 6b). Similarly, GSH content was not affected when Caco-2 was exposed to Clay 2 (Fig. 7a) In contrast, GSH content significantly ($p<0.01$) depleted in HepG2 cells, especially from 44 µg/mL onwards. The percentage of GSH for 44 µg/ml was 8 fold lower in comparison to the control group (Fig. 7b). ~~** p<0.01 significantly different from control.~~

3.3. IL-6 leakage

The IL-6 assay showed no increase in the IL-6 content in the cell culture medium of the cytotoxicity assays in any of the treatments performed (data not shown).

3.4. Comet assay

Cloisite®20A and Clay 1 did not induce DNA strand-breaks in Caco-2 and Hep-G2 cells at the concentrations tested after 24 and 48h of exposure (Fig. 8 and 9). In regard to Caco-2 cells exposed to 8.5 and 17 µg/mL of Clay 2, no significant variations of the DNA strand breaks were detected after 24 h and 48 h of exposure. However, the highest exposure concentration (34 µg/mL) led to a significant ($p<0.05$) increase of DNA in the tails, compared to the control (Fig. 10a). The percentage of DNA in the tail with this concentration was not significantly different from the results obtained for the positive control H_2O_2~~(* p<0.05 significantly different from control)~~. In treated HepG2 cells a significant ($p<0.01$) increase of DNA damage was observed after 24h exposure at the highest concentration only (88 µg/mL). In contrast, after 48h, a significant increase of DNA strand breaks was detected in cells exposed to 44 ($p<0.05$) and 88 ($p<0.01$) µg/mL (Fig. 10b). The percentage of DNA in the tail for 44 and 88 µg/mL was 3.5 and 13 fold higher respectively, in comparison to the control group. ~~* p<0.05 **p<0.01 significantly different from control.~~

4. Discussion

The use of modified clays is a great option to obtain nanocomposites with improved properties that have been shown to be very useful in food packaging applications. Massive quantities of nanomaterials would need to be produced, thereby increasing the potential risk of human exposure and raising additional concern about their short and long term toxicological effects (Hussain et al., 2009). Due to this lack of information, a specific toxicity assessment of these materials and their precursors (non modified and chemically organomodified clays) is necessary. Therefore, in the present study, biochemical alterations with three different montmorillonite-based clays, all of them chemically modified with quaternary ammonium salts; a commercial one, Cloisite®20A, and two developed by ITENE, Clay 1 and Clay 2, were studied in the human cell lines Caco-2 and HepG2. The results obtained showed that, at the concentrations assayed, the main cytotoxic effects are induced by Clay 2 with a time-dependent pattern. Nevertheless, the toxicity assessment of Cloisite®20A and Clay 1 showed no toxic (cytotoxic and genotoxic) effects at the concentrations assayed, although they were lower than those used for Clay2.In this sense, the importance of the modifiers that are used to improve the compatibility with the polymer matrix must be highlighted. The incorporation of ACO in Clay 2 structure could be related with the different toxicity profile of Clay 1 and Clay 2 observed in both cell lines. ACO provides a lower hydrophobicity to the clay that allows testing higher concentrations in the aqueous culture medium. When the same test concentration (8 µg/mL) of both clays is compared in Caco-2 cells, results are quite similar with a low decrease of viability. In HepG2 on the other hand, 8 µg/mL Clay 1 induced a significant decrease of protein content, but 8 µg/mL Clay 2 did not induce any effect. Unfortunately, higher concentrations of Clay 1 could be not assayed due to the presence of interferences with the measurement system. A similar comparison performed with the same concentration (62.5 µg/ml) of Clay 2 and Cloisite®20A shows the higher toxicity of Clay 2.

344 Others authors have evaluated the toxicity of the commercial non modified montmorillonite in the
345 same cell lines (Gutierrez- Praena et al., 2011; Lordan et al., 2011; Maisanaba et al., 2013a,c;
346 Sharma et al., 2010). The toxicity assays carried out by Lordan et al. (2011) with the commercial
347 unmodified clay, designated as Cloisite®Na+ showed a significant cell viability decrease in all the
348 concentrations assayed, from 1 to 1000 µg/mL, in the HepG2 cell line. These results do not
349 agree with Maisanaba et al. (2013a,c), who observed that HepG2 and Caco-2 exposed to
350 Cloisite®Na^+ did not present higher significant reductions of viability with respect to the controls in
351 the range of concentrations assayed, from 0-62.5 and 0-125 µg/mL, after 24 and 48h of
352 exposure, respectively. Moreover, Sharma et al. (2010), did not obtain any cytotoxic effects in
353 Caco-2 exposed to Cloisite®Na^+, in agreement with our results. In this case, the concentration
354 range used of the modified clays Cloisite®20A and Clay 1 showed the same behaviour compared
355 to the starting material, unmodified montmorillonite, indicating that the modifiers employed could
356 not involve changes in the safety profile of the modified clays.
357 There are a limited number of toxicological studies in the literature about commercial modified
358 clays in Caco-2 and HepG2. Lordan et al. (2011) also evaluated a commercial modified clay,
359 Cloisite®93A, in the hepatic cell line, obtaining cytotoxic effects at all concentrations tested (1-
360 1000µg/mL). Other commercial modified montmorillonite, Cloisite®30B, has been evaluated in
361 Caco-2 cells by Sharma et al. (2010). The research group evaluated filtered and unfiltered
362 suspensions of Cloisite®30B. The results showed a notably cytotoxic effect at the highest
363 concentrations assayed in both cases, reporting a 40% cell viability reduction at 226 µg/mL. The
364 cytotoxic effects of this modified clay in Caco-2 and HepG2 have been also studied by our
365 research group, obtaining cytotoxicity for all the biomarkers assayed, being the highest
366 concentration assayed 250 and 500 µg/mL, respectively (Maisanaba et al., 2013a,c).
367

368 The results obtained showed that the intestinal cell line, Caco-2 was more sensitive in cytotoxicity
369 parameters to the modified clays exposure. Thus, in the present study the EC50 (24h) for the PC
370 assay calculated after Clay 2 exposure was 93 µg/mL for HepG2 and 34 µg/mL for Caco-2.
371 These results agree with those obtained by the other authors, that also observed a higher
372 sensitivity after exposure to Cloisite®30B in Caco-2, with an EC50 = 40µg/mL (Maisanaba et al.,
373 2013c) versus an EC50 = 88 µg/mL for HepG2 (Maisanaba et al., 2013a). However, biomarkers
374 showed a different sensitivity in both cell lines, the PC was the most sensitive in Caco-2 whereas
375 MTS was the most sensitive in HepG2. This could be related with their different origin and
376 therefore to their different capacity to face toxic insults.
377
378 Others authors have studied the cytotoxic effects of commercial clays in other target cell lines.
379 Baek et al. (2012) evaluated the toxicity effects in human normal intestinal cells (INT-407) in a
380 short and long term exposure, 24, 48, 72h, and, 10 days, to MMT. Thereby, a decrease in cell
381 proliferation showed at all times assayed. On the one hand, significant differences in the short
382 term assays were found above 100 µg/mL concentration levels, on the other hand, a significant
383 inhibition of normal colony formation in the long term was observed at all concentrations tested.
384 Even though, alterations in LDH release were only observed at the highest concentrations at 48
385 and 72h. Li et al. (2010) also studied CHO cell viability, when exposed to 62.5 – 1000 µg/mL
386 nanosilicate platelets, obtaining a slight decrease in MTT and LDH assays. Also, the oligo
387 (styrene- co- acrylonitrile)- modified clay montmorillonite showed an increased LDH release
388 activity and cell viability reduction at a concentration of 1g/L in mouse embryonic fibroblast (NIH
389 3T3) cells and human embryonic kidney 293 (HEK 293) cells (Liu et al., 2011).
390
391 Taking into account that Clay 2 showed a clear cytotoxic profile at the concentration range tested
392 in comparison to Closite®20A and Clay 1, the toxic mechanisms of this organoclay were

393 considered to require further study. The implication of oxidative stress, inflammation or DNA
394 damage, among others, could be related to micro and nanoparticles exposure (Bouwmeester et
395 al., 2009). For this reason, levels of ROS and GSH were assayed in this study, obtaining only
396 significant differences with respect to the control group on the GSH levels of HepG2. The results
397 showed therefore a higher sensitivity of the HepG2 cell line to oxidative stress biomarkers in
398 contrast to the results obtained in the cytotoxicity study. Other authors have previously reported
399 that HepG2 cells are generally more susceptible to oxidant-induced stress than Caco-2 cells
400 (Martin et al., 1997). Our research group has also observed a deep decrease in GSH content in
401 HepG2 exposed to Cloisite®30B at all timepoints assayed, however no changes were observed
402 in ROS levels (Maisanaba et al., 2013a). This might be due because GSH have many biological
403 functions apart from ROS scavenging such as signal transduction, gene expression and
404 apoptosis, etc. (Sies 1999). Moreover, Sharma et al. (2010) also reported that Closite®Na^+ and
405 Closite®30B did not induce ROS production in Caco-2, in agreement with our results for those
406 clays (Maisanaba et al., 2013a) and although there was no change in ROS levels they observed
407 genotoxic effects. By contrast, Lordan et al. (2011) reported that Cloisite®Na^+ induced
408 intracellular ROS formation, whereas, the effect of Cloisite®93A in the generation of ROS was
409 less prominent. Furthermore, Baek et al. (2012) evaluated the ROS production in INT-407 cells
410 exposed to MMT, obtaining significant levels of ROS at the highest concentration (1000 µg/ mL)
411 at all three timepoints assayed (24, 48 and 72h).
412
413 Clay 2 did not induce leakage of IL-6, biomarker of an inflammatory response, in any of the cell
414 lines. MMT, on the other hand, has been reported to rapidly lyses neutrophils and erythrocytes in
415 vitro. Furthermore, it can stimulate chemiluminescence, the neutrophil oxidative metabolic burst
416 (Dougherty et al., 1985).
417

418 In relation to genotoxicity, Clay 2 induced DNA strand breaks in both cell lines Moreover, Clay 2
419 induced time-dependent increases of DNA strand breaks in HepG2 cells. Similar results were
420 also found by Maisanaba et al., (2013a) for Cloisite30®B in HepG2 cells. Other authors evaluated
421 the genotoxicity of other commercial clays in similar cell lines. Sharma et al. (2010), showed no
422 DNA damage in Caco-2 exposed to unfiltered and filtered Cloisite®Na^+ samples. Nevertheless,
423 DNA damage was observed in the intestinal cell line Caco-2 exposed to Cloisite®30B from 113
424 µg/mL after 24h. By contrast, the results obtained by Maisanaba et al., (2013c) with the intestinal
425 cell line exposed to Cloisite®30B, did not coincide with the aforementioned research group, and
426 this can be related with the lower concentrations tested in this case (10, 20 and 40 µg/mL).
427 Maisanaba et al. (2013a), however, showed differences with respect to the control at the highest
428 concentration assayed (88 µg/mL) in HepG2 after 48 h. In this case, it is difficult to compare
429 because Sharma et al. (2010) only presented results after 24h, and at this concentration DNA
430 damage in the intestinal cell line was not observed.
431 Sharma et al. (2010), also indicated that the modifiers used to modify the clay contributed to the
432 genotoxic effects, and these effects were not due to oxidative damage. In this sense, the absence
433 of ROS obtained in this work after exposure to Clay 2 coincides with the Sharma´s hypothesis.
434 On the other hand, Li et al. (2010) observed no genotoxic results in the comet assay with CHO
435 cells exposed up to 1000µg/mL nanosilicate platelets derived from MMT.
436 Regarding to in vivo toxicity data, there is no information about the three organoclays selected in
437 the present study, but Li et al. (2010) did not reported acute oral toxicity of MMT in rats exposed
438 to a single dose of 5700 mg/kg. Also Baek et al. (2012) did not find any remarkable toxicity in
439 mice orally exposed to a single dose of 1000 mg/kg MTT.
440
441 In summary, our results showed that at the concentrations assayed Cloisite®20A and Clay 1 did
442 not induce cytotoxicity. Clay 2, however, showed cytotoxic and genotoxic effects, as well as an

alteration in GSH content. Therefore, a case by case toxicological assessment is required, as the modifier has a role in the toxicity observed. Moreover, a different sensitivity was observed depending on the cell line employed.

More studies are required to elucidate the risks of these clays for human health as their use is going to increase widely in the near future.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, and the Cell Culture Service and Microscopy Service of Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS) for providing technical assistance.

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577 **Table captions**

578 Table 1. EC50 values (µg/mL) obtained after 24 and 48h exposure to Clay 2 in both cell lines.

579

580 **Figure captions**

581 Figure 1. TGA results for Cloisite®Na^+ (round) and Cloisite®20A (square).

582

583 Figure 2. FTIR results for Cloisite®Na^+ (up) and Cloisite®20A (down).

584

585 Figure 3. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
586 HepG2 (b,d) cells after 24h and 48h of exposure to 0-8 µg/mL Clay 1. Results from 3 independent
587 experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences
588 were considered significant from $p<0.05$ (*).

589

590 Figure 4. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
591 HepG2 (b,d) cells after 24h and 48h of exposure to 0-125 µg/mL Clay 2. Results from 3
592 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d.
593 Differences were considered significant from $p<0.05$ (*).

594

595 Figure 5. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
596 HepG2 (b,d) cells after 24h and 48h of exposure to 0-62.5 µg/mL Cloisite®20A. Results from 3
597 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d.
598 Differences were considered significant from $p<0.05$ (*).

599

600 Figure 6. ROS content (a) in Caco-2 cells after 24 and 48h of exposure to 8.5, 17, or 34 µg/mL
601 Clay 2. ROS content (b) in HepG2 cells after 24 and 48h of exposure to 22, 44, or 88 µg/mL Clay

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2. Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d.

Figure 7. GSH content (a) in Caco-2 cells after 24 and 48h of exposure to 8.5, 17, or 34 µg/mL Clay 2. GSH content (b) in HepG2 cells after 24 and 48h of exposure to 22, 44, or 88 µg/mL Clay 2. Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d. ** significantly different from control ($p < 0.01$).

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Figure 8. Comet assay results (a) of Caco-2 -and (b) HepG2 cells after 24 and 48h of exposure to 15.65, 31.25, or 62.5 µg/mL Cloisite®20A. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$).

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Figure 9. Comet assay results (a) of Caco-2 and (b) HepG2 cells after 24 and 48h of exposure to 2, 4 or 8 µg/mL Clay 1. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$).

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Figure 10. Comet assay results (a) of Caco-2 cells after 24 and 48h of exposure to 8.5, 17-, or 34 µg/mL Clay 2. Comet assay results (b) of HepG2 cells after 24 and 48h of exposure to 22, 44 and 88µg/mL. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$). ** significantly different from control ($p \leq 0.01$).

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Highlights:

- Organomodified clays used in packaging induce cytotoxic effects in target cell lines.
- Clay 2 induces cyto and genotoxicity in both cell lines assayed.
- Clay 2 induces GSH decrease in HepG2.
- The modifiers of the clays could be related with their toxicity profile.

Toxicity assessment of organomodified clays used in food contact materials on human target cell lines

Houtman J[1], Maisanaba S[2], Puerto M[2*], Gutiérrez-Praena D[2], Jordá M[3], Aucejo S[3], Jos A[1]

[1]Wageningen University and Research Center. Wageningen. The Netherlands.

[2]Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[3]Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

*Corresponding author:

María Puerto

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: mariapuerto@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Nowadays, the incorporation of organomodified clays based on montmorillonite into polymers intended for packaging industry is a reality. The final result is a polymer nanocomposite with enhanced barrier properties. Different organomodified clays are already commercially available and others new ones are being developed, however little is known about their safety.

In the present work, the cytotoxic effects (a tetrazolium salt reduction and protein content) of three organomodified clays, Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified clays developed by the Packaging, Transport, & Logistics Research Institute, were evaluated in Caco-2 and HepG2 cells after 24 and 48h of exposure. Our results showed that only Clay 2 induced toxic effects in both cell lines. The mean effective concentration was calculated for each case, showing Caco-2 to be more sensitive than HepG2. Moreover, in order to elucidate the toxicity mechanisms of Clay 2, different mechanistic biomarkers were investigated. Interleukin leakage and generation of intracellular reactive oxygen species was not observed, whereas glutathione content decreased in HepG2. DNA damage (comet assay) was induced in both cell lines at the highest concentration tested. Overall, results show that the type of clay, the concentrations range and the type of cell line play an important role in the toxicity observed.

Keywords: clay, montmorillonite, cytotoxicity, cell lines, DNA damage

1. Introduction

Over the last decades, the use of polymers as food packaging materials has increased enormously due to their advantages over other traditional materials (Jordan et al., 2005; Silvestre et al., 2011). Latest innovations include the use of "improved" food contact materials, this is the addition of different reinforcements, for example nanoparticles, to the polymeric matrix in order to enhance the functional properties of packaging materials, and thus improve the shelf life of food and beverage products. The resulting nanocomposites are a multi-phase material in which the majority of the dispersed phase components have one or more dimensions of the order of 100 nm or less (SCENIHR, 2007).

Smectite clays, such as montmorillonite (MMT), belong to the structural family called 2:1 phyllosilicates, which present a structure composed by two tetrahedral layers formed by Si and O atoms, fused with an octahedral layer with aluminum and magnesium atoms bonded to oxygen and hydroxyl groups (Jordá-Beneyto et al., 2008). They are one of the main choices for designing polymer nanocomposites due to their low cost and rich intercalation chemistry allowing them to be chemically modified (organoclays) and to improve the compatibility with the polymer matrix (Bitinis et al., 2011). Once the final clay nanocomposites are ready, the resulting material presents a specific disposition of the clays, such as platelets, giving a tortuous path to the gas permeant, forcing it to travel a longer path to diffuse through the film (Nielsen, 1967). Several authors reviewed the advantages, and in few cases the limitations, of the layered nanocomposites. Great improvements in thermal, mechanical and barrier (permeability) properties are presented, as well as strength, stiffness, dimensional stability, and heat resistance (De Azeredo, 2009; Duncan et al., 2011; Hatzigrigoriou et al., 2011; Hetzer et al., 2008). Moreover, they also offer good barrier properties under different packing, handling, shipping, and

storage conditions (Avella et al., 2005; Brody, 2006; Ray et al., 2006; Timoty and Duncan 2011; Volpe, 2005), therefore they could improve the quality and safety of packaged food (Lagaron,2006).

Although there is a lot of evidence for the good technological performance of nanocomposites, safety issues are also of importance. Available data on clay's toxicity is still scarce, but different authors have already described toxic effects induced by montmorillonite and organoclays (Lordan et al., 2011; Maisanaba et al., 2013a,c; Sharma et al., 2010). Moreover, the modifier used to synthesize the organoclay has a role in the toxicity observed (Maisanaba et al., 2013a).

In the case of organoclays, the oral pathway is the most important entrance route for the consumers, as they are exposed to the possible migrants from packaging nanocomposites to the food products, and they should know the possible effects of the ingestion of these nanosubstances to the gastrointestinal tract (Silvestre et al., 2011). When the nanoparticles reach the blood circulation, the liver is among the main organs for distribution (SCENIHR, 2009), so for clays imbued in the nanocomposite material this possibility cannot be discarded.

Taking into account that the production of nanocomposites is going to increase in the near future, the human exposure will also increase. Therefore, potential toxic effects should be investigated in order to guarantee the safety of the food products.

The aim of this study is to evaluate the toxicity of three modified montmorillonite clays used in the production of nanocomposites with applications as food contact materials in two target human cell lines of the digestive system, Caco-2 and HepG2 from intestinal and hepatocellular origin, respectively. For this issue, basal cytotoxicity biomarkers and mechanistic biomarkers of

inflammation, oxidative stress and genotoxicity have been investigated with Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified organoclays developed by the Technological Institute of Packaging, Transport and Logistics (ITENE).

2. Materials and Methods

2.1. Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Clays

Clay1 and Clay2 are two novel microsized clays that have been developed and characterized by Thermogravimetric Analysis (TGA) and Fourier Transform InfraRed (FTIR), as described in Maisanaba et al, (2013b) and Jordá-Beneyto et al., (2008, 2013).Clay 1 contains as modifier quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) and Clay 2 contains HDTA and acetylcholine chloride (ACO). Both clays are obtained by cation exchange reaction from Cloisite®Na^+ (Southern Clay Products, INC.). This raw clay has a typical dry particle size less than 25μm (d50). Cloisite®20A was also obtained from Southern Clay Products, INC. (modifier: dimethyl, dehydrogenated tallow, quaternary ammonium, concentration: 95 meq/100g clay). This modified clay has a typical dry particle size less than 10μm (d50) and has been characterized by Thermogravimetric Analysis (TGA) and Fourier Transform InfraRed (FTIR).

TGA analysis of Cloisite®20A and Cloisite®Na^+ (the original non-modified clay included as control) were performed on a Q5000IR thermobalance (TA Instruments) by heating the samples

119 from room temperature up to 900 °C with heating rate of 10 °C/min, in nitrogen atmosphere.
120 Approximately 7 mg of each finely ground sample was heated in a platinum crucible.
121
122 FTIR Spectra were obtained on an Equinox 55 spectrometer (Bruker), coupled to a microscope
123 modulus with ATR objetive (Hiperion, Bruker). This technique was used to characterize both clays
124 Cloisite®Na^+ and Cloisite®20A. For each sample 128 scans were recorded with a resolution of 4
125 cm^{-1}.
126
127 2.3. Model systems
128 Caco-2 cell line derives from a human colon carcinoma (ATCC® HTB-37) and HepG2 is a human
129 hepatocellular carcinoma epithelial cell line (ATCC® HB-8065). Both of them were obtained from
130 the American Type Culture Collection. Caco-2 cell line was maintained in Eagle's medium
131 (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50
132 µg/mL gentamicine, 2 mM L-glutamine, and 1 mM pyruvate. HepG2 cell line was maintained in
133 EMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells
134 were grown near confluence in 75-cm^2 plastic flasks at 37ºC in an atmosphere containing 5%
135 CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25%
136 trypsin. They were counted in an improved Neubauer haemocytometer and viability was
137 determined by the Trypan Blue exclusion test. The cells were used at passages between 10 and
138 21. Both cell lines were plated at a density of 7.5 x 10^5 cells/mL to perform all experiments.
139
140 2.4. Clays test solutions
141 Test concentrations of clays were determined individually in previous experiments in order to
142 avoid interference with the method of measurement. With this purpose the absorbance of clay
143 solutions (1000 µg/mL and serial ½ dilutions) were measured at 0, 24 and 48h. The

concentrations selected were the highest ones that did not show statistical differences versus the control. Thus, the maximum concentrations were 8 µg/mL for Clay 1, 125 µg/mL for Clay 2, and 62.5 µg/mL for Cloisite®20A. Test solutions were prepared in serum-free medium. An ultrasonic tip (Dr. Hielscher, Germany) at an amplitude of 40% for a total time of 30 s was employed to disperse the test concentrations.

2.5. Cytotoxicity assays

From the initial solutions, serial dilutions in medium without serum were prepared. Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37ºC for 24 and 48 hours. The basal cytotoxicity endpoints assayed were tetrazolium salt reduction (MTS) and protein content (PC).

MTS reduction is carried out by dehydrogenases, enzymes present in mitochondria, making this endpoint a good marker for the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium salt) added to the medium is reduced by the enzymes to a colored formazan product soluble in culture medium and is measured by a spectrophotometer at 490 nm after 2 hours of incubation in the dark.

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage with independence of the toxic mechanism involved (Pichardo et al. 2007). PC was quantified *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the total cell number present in the wells. The culture medium was replaced by

200 µL NaOH and after 2 h of incubation at 37ºC, 180 µL were replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm in a microplate spectrophotometer (Tecan Infinite M200, Austria).

2.6. Oxidative stress assays

Considering that only Clay 2 showed remarkable cytotoxic effects, this clay was chosen to perform mechanistic studies. For this purpose low cytotoxic concentrations were selected: 0, 8.5, 17 and 34 µg/mL for Caco-2 cells and 22, 44 and 88 µg/mL for HepG2 cultures. These concentrations correspond to the mean effective concentration (EC_{50}) value obtained for the most sensitive cytotoxicity endpoint at 24h along with the fractions $EC_{50}/2$ and $EC_{50}/4$. After replacing the previous medium, the exposure solutions were added to the cells, and incubated at 37ºC for 24 and 48 hours. Culture medium without clay was used as control group. The oxidative stress endpoints measured were reactive oxygen species (ROS) content and glutathione (GSH) levels.

The production of ROS was assessed in 96 well microplates using the dichlorofluorescein (DCF) assay. The probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Invitrogen) readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent compound (DCFH), which is rapidly oxidized in the presence of ROS to the highly fluorescent DCF. Specifically, cells were incubated with 200 µl 20 µM DCFH-DA in culture medium at 37ºC for 30 min, and then washed with phosphate buffered saline (PBS) and resuspended in 200 µl of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as fluorescence arbitrary units (Puerto et al. 2010).

GSH content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos et al. 2009). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme GST. After the cell exposure to the clay, medium was discarded and cells were incubated at 37°C for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the excitation/emission wavelengths of 380/460 nm. Results were expressed as arbitrary units.

2.7. Interleukin-6 leakage

For this assay, the culture medium of the cells after 24 and 48h exposure to Clay 2 was used. Manufacturer instructions from the kit (EH2IL6, Thermo Scientific, USA) were followed.

2.8. Comet assay

The comet assay was performed to detect DNA strand breaks. The cells lines were seeded into 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37ºC in 5% CO_2 to attach to the plates. Approximately 3.5×10^5 Caco-2 or HepG2 cells were exposed to different concentrations of Clay 2: 0, 8.5, 17, 34 µg/ml and 0, 22, 44 an 88 µg/ml, respectively, for 24 and 48 h.

In order to monitor the ongoing process of the assay, a negative control (cells treated with culture medium) and a positive control (cells treated with a solution of 100 µM H_2O_2) were included. After treatments cells were washed and detached in PBS. The comet assay was applied as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, cells were resuspended in PBS at a concentration of $2.5x10^6$ cells/mL. This suspension was mixed with 1% low melting point agarose and placed on a microscope slide (12 gels per slide). Once the gels

219 became solid, the slides were dipped into lysis solution at 4ºC. All nucleotides were denatured in
220 a high-pH buffer. Electrophoresis was carried out at approximately 25 V (300 mA, ~ 1V/cm),and
221 the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope
222 slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold
223 nucleic acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20×
224 objective) coupled via a CCD camera to an image-analysis system (DP controller-DP manager).
225 Images of randomly selected nuclei (≥100) per experimental point were analyzed with the image
226 analysis software (Comet Assay IV, Perceptive Instruments, UK). The results from four
227 independent experiments are expressed as % of tail DNA.
228
229 2.9. Calculations and statistical analysis
230 All experiments were performed at least three times and at least in duplicate per concentration
231 (n=3). Data for the concentration-dependent cytotoxicity relationships of all experiments were
232 presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control.
233 Statistical analysis was carried out with GraphPad InStat3 software (La Jolla, CA, USA) using
234 analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. This test
235 compares the response of the different exposure concentrations versus the controls. Previous to
236 ANOVA, normality was checked with Kolmogorov–Smirnov test (p>0.05) and variance
237 homogeneity among groups assessed with Bartlett's homocedasticity test (p>0.05). Differences
238 among groups were considered significant at p<0.05.
239 EC_{50} values (mean effective concentration, concentration that modified each biomarker by 50%,
240 positive or negative, in comparison with appropriate untreated controls) were derived by linear
241 regression in the concentration-response curves.
242
243 **3. Results**

244 3.1. Characterization of Cloisite®20A

245 TGA results of both clays (Cloisite®Na^+ as control and Cloisite®20A) are presented in Figure 1.
246 Loss weight percent (%), and derivative weight (%/°C) are presented versus temperature. It can
247 be observed that only the sample Cloisite®20A has a big loss step in the range between 200 and
248 500 ºC, indicating that an organic compound is decomposed. The percentage of organic modifier
249 in Cloisite®20A is 24.87 % w/w, as calculated in the TGA curves. Cloisite®Na^+ hardly presents
250 any weight change in this range of temperature.

251 The presence of modifiers in the clays was determined by FTIR. The ATR spectra are shown in
252 Figure 2. Characteristic Si-O stretching vibration band of the unmodified montmorillonite (cloisite Na+)
253 occurs at 1030 cm^{-1}, whereas in the spectra of the corresponding organoclay (cloisite20A) the band
254 appears slightly shifted (1000 cm^{-1})(Madejova, 2003). Inner hydroxyl groups, lying between the
255 tetrahedral and octahedral sheets, gives the absorption near 3620 cm^{-1}, as it can be seen in both
256 spectra in Figure 2. The C-H stretching bands of alkylammnium cations occur in the range of
257 3020-2800 cm^{-1}. The C-H stretching bands at 2930 cm^{-1} and at 2850 cm^{-1} are present in spectra
258 of Cloisite®20A, which is a organo-modified clay. A characteristic C-H bending vibration of the
259 $(CH3)4N^+$ cation at 1487 cm^{-1} is seen in the spectrum of the modified clay, which corresponds
260 with the alkylammonium modifier in this clay. The spectrum of Cloisite®Na^+ does not present
261 these peaks, showing that in this clay there is no presence of any organic modifier.

262

263 3.2. Cytotoxicity assays

264 MTS reduction performed by Caco-2 and HepG2 cells exposed to Clay 1 remained unaltered with
265 respect to the control cells after 24 and 48 h (Fig. 3a & 3b). In respect to PC, Caco-2 cell line
266 showed no alterations at 24 h, whereas at 48 h only experienced a significant (p<0.05) decrease
267 respect to the control at 8 µg/mL (Fig. 3c). In the case of HepG2 cells, PC did not show

268 significant changes with respect to the control after both exposure times, except at the highest
269 concentration assayed in both cases (Fig. 3d).
270
271 On the other hand, the MTS assay with Clay 2 presented a significant (p<0.05) decrease from 30
272 µg/mL at both exposure times in Caco-2 cell line (Fig. 4a). Similarly, MTS reduction in HepG2
273 cells presented a decrease from the concentration of 62.5 µg/mL after both exposure times, with
274 significant differences at 48h (Fig. 4b). Moreover, Caco-2 cells showed a concentration and time-
275 dependent decrease in PC, with significant (p<0.05) reductions in cell viability from10 µg/mL
276 onwards in cells exposed for 24 h and from 5µg/mL onwards after 48 h of exposure (Fig. 4c).
277 Concerning to HepG2 cells, significant (p<0.05) changes appeared at the highest concentration
278 assayed after 24 h, and from 62.5 µg/mL after 48 h (Fig. 4d).
279
280
281 Regarding to Cloisite®20A, both cytotoxicity biomarkers assayed in Caco-2 and HepG2 cell lines,
282 MTS (Fig. 5a & 5b) and PC (Fig. 5c & 5d), remained unaltered at any concentration assayed in
283 respect to control cells after 24 and 48 h.
284
285 EC_{50} values could be obtained for Clay 2 only, showing a higher toxicity at the longest exposures
286 (Table 1). Moreover, these data indicate that, in relation to cytotoxicity, Caco-2 cells have a
287 higher sensitivity than HepG2 cells, as EC_{50} values are lower.
288
289 *3.2.* Oxidative stress assays
290 When Caco-2 or HepG2 cells were exposed to Clay 2 during 24 and 48 h, no significant alteration
291 on ROS was observed at any of the exposure concentrations in comparison to the control group
292 (Fig. 6a & 6b). Similarly, GSH content was not affected when Caco-2 was exposed to Clay 2 (Fig.

7a) In contrast, GSH content significantly (p<0.01) depleted in HepG2 cells, especially from 44 µg/mL onwards. The percentage of GSH for 44 µg/ml was 8 fold lower in comparison to the control group (Fig. 7b).

3.3. IL-6 leakage

The IL-6 assay showed no increase in the IL-6 content in the cell culture medium of the cytotoxicity assays in any of the treatments performed (data not shown).

3.4. Comet assay

Cloisite®20A and Clay 1 did not induce DNA strand-breaks in Caco-2 and Hep-G2 cells at the concentrations tested after 24 and 48h of exposure (Fig. 8 and 9). In regard to Caco-2 cells exposed to 8.5 and 17 µg/mL of Clay 2, no significant variations of the DNA strand breaks were detected after 24 h and 48 h of exposure. However, the highest exposure concentration (34 µg/mL) led to a significant (p<0.05) increase of DNA in the tails, compared to the control (Fig. 10a). The percentage of DNA in the tail with this concentration was not significantly different from the results obtained for the positive control H_2O_2. In treated HepG2 cells a significant (p<0.01) increase of DNA damage was observed after 24h exposure at the highest concentration only (88 µg/mL). In contrast, after 48h, a significant increase of DNA strand breaks was detected in cells exposed to 44 (p<0.05) and 88 (p<0.01) µg/mL (Fig. 10b). The percentage of DNA in the tail for 44 and 88 µg/mL was 3.5 and 13 fold higher respectively, in comparison to the control group.

4. Discussion

The use of modified clays is a great option to obtain nanocomposites with improved properties that have been shown to be very useful in food packaging applications. Massive quantities of nanomaterials would need to be produced, thereby increasing the potential risk of human exposure and raising additional concern about their short and long term toxicological effects (Hussain et al., 2009). Due to this lack of information, a specific toxicity assessment of these

318 materials and their precursors (non modified and chemically organomodified clays) is necessary.
319 Therefore, in the present study, biochemical alterations with three different montmorillonite-based
320 clays, all of them chemically modified with quaternary ammonium salts; a commercial one,
321 Cloisite®20A, and two developed by ITENE, Clay 1 and Clay 2, were studied in the human cell
322 lines Caco-2 and HepG2. The results obtained showed that, at the concentrations assayed, the
323 main cytotoxic effects are induced by Clay 2 with a time-dependent pattern. Nevertheless, the
324 toxicity assessment of Cloisite®20A and Clay 1 showed no toxic (cytotoxic and genotoxic) effects
325 at the concentrations assayed, although they were lower than those used for Clay2.In this sense,
326 the importance of the modifiers that are used to improve the compatibility with the polymer matrix
327 must be highlighted. The incorporation of ACO in Clay 2 structure could be related with the
328 different toxicity profile of Clay 1 and Clay 2 observed in both cell lines. ACO provides a lower
329 hydrophobicity to the clay that allows testing higher concentrations in the aqueous culture
330 medium. When the same test concentration (8 µg/mL) of both clays is compared in Caco-2 cells,
331 results are quite similar with a low decrease of viability. In HepG2 on the other hand, 8 µg/mL
332 Clay 1 induced a significant decrease of protein content, but 8 µg/mL Clay 2 did not induce any
333 effect. Unfortunately, higher concentrations of Clay 1 could be not assayed due to the presence
334 of interferences with the measurement system. A similar comparison performed with the same
335 concentration (62.5 µg/ml) of Clay 2 and Cloisite®20A shows the higher toxicity of Clay 2.

336 Others authors have evaluated the toxicity of the commercial non modified montmorillonite in the
337 same cell lines (Gutierrez- Praena et al., 2011; Lordan et al., 2011; Maisanaba et al., 2013a,c;
338 Sharma et al., 2010). The toxicity assays carried out by Lordan et al. (2011) with the commercial
339 unmodified clay, designated as Cloisite®Na+ showed a significant cell viability decrease in all the
340 concentrations assayed, from 1 to 1000 µg/mL, in the HepG2 cell line. These results do not
341 agree with Maisanaba et al. (2013a,c), who observed that HepG2 and Caco-2 exposed to
342 Cloisite®Na^+ did not present higher significant reductions of viability with respect to the controls in

343 the range of concentrations assayed, from 0-62.5 and 0-125 µg/mL, after 24 and 48h of
344 exposure, respectively. Moreover, Sharma et al. (2010), did not obtain any cytotoxic effects in
345 Caco-2 exposed to Cloisite®Na^+, in agreement with our results. In this case, the concentration
346 range used of the modified clays Cloisite®20A and Clay 1 showed the same behaviour compared
347 to the starting material, unmodified montmorillonite, indicating that the modifiers employed could
348 not involve changes in the safety profile of the modified clays.
349 There are a limited number of toxicological studies in the literature about commercial modified
350 clays in Caco-2 and HepG2. Lordan et al. (2011) also evaluated a commercial modified clay,
351 Cloisite®93A, in the hepatic cell line, obtaining cytotoxic effects at all concentrations tested (1-
352 1000µg/mL). Other commercial modified montmorillonite, Cloisite®30B, has been evaluated in
353 Caco-2 cells by Sharma et al. (2010). The research group evaluated filtered and unfiltered
354 suspensions of Cloisite®30B. The results showed a notably cytotoxic effect at the highest
355 concentrations assayed in both cases, reporting a 40% cell viability reduction at 226 µg/mL. The
356 cytotoxic effects of this modified clay in Caco-2 and HepG2 have been also studied by our
357 research group, obtaining cytotoxicity for all the biomarkers assayed, being the highest
358 concentration assayed 250 and 500 µg/mL, respectively (Maisanaba et al., 2013a,c).
359
360 The results obtained showed that the intestinal cell line, Caco-2 was more sensitive in cytotoxicity
361 parameters to the modified clays exposure. Thus, in the present study the EC50 (24h) for the PC
362 assay calculated after Clay 2 exposure was 93 µg/mL for HepG2 and 34 µg/mL for Caco-2.
363 These results agree with those obtained by the other authors, that also observed a higher
364 sensitivity after exposure to Cloisite®30B in Caco-2, with an EC50 = 40µg/mL (Maisanaba et al.,
365 2013c) versus an EC50 = 88 µg/mL for HepG2 (Maisanaba et al., 2013a). However, biomarkers
366 showed a different sensitivity in both cell lines, the PC was the most sensitive in Caco-2 whereas

MTS was the most sensitive in HepG2. This could be related with their different origin and therefore to their different capacity to face toxic insults.

Others authors have studied the cytotoxic effects of commercial clays in other target cell lines. Baek et al. (2012) evaluated the toxicity effects in human normal intestinal cells (INT-407) in a short and long term exposure, 24, 48, 72h, and, 10 days, to MMT. Thereby, a decrease in cell proliferation showed at all times assayed. On the one hand, significant differences in the short term assays were found above 100 µg/mL concentration levels, on the other hand, a significant inhibition of normal colony formation in the long term was observed at all concentrations tested. Even though, alterations in LDH release were only observed at the highest concentrations at 48 and 72h. Li et al. (2010) also studied CHO cell viability, when exposed to 62.5 – 1000 µg/mL nanosilicate platelets, obtaining a slight decrease in MTT and LDH assays. Also, the oligo (styrene- co- acrylonitrile)- modified clay montmorillonite showed an increased LDH release activity and cell viability reduction at a concentration of 1g/L in mouse embryonic fibroblast (NIH 3T3) cells and human embryonic kidney 293 (HEK 293) cells (Liu et al., 2011).

Taking into account that Clay 2 showed a clear cytotoxic profile at the concentration range tested in comparison to Closite®20A and Clay 1, the toxic mechanisms of this organoclay were considered to require further study. The implication of oxidative stress, inflammation or DNA damage, among others, could be related to micro and nanoparticles exposure (Bouwmeester et al., 2009). For this reason, levels of ROS and GSH were assayed in this study, obtaining only significant differences with respect to the control group on the GSH levels of HepG2. The results showed therefore a higher sensitivity of the HepG2 cell line to oxidative stress biomarkers in contrast to the results obtained in the cytotoxicity study. Other authors have previously reported that HepG2 cells are generally more susceptible to oxidant-induced stress than Caco-2 cells

392 (Martin et al., 1997). Our research group has also observed a deep decrease in GSH content in
393 HepG2 exposed to Cloisite®30B at all timepoints assayed, however no changes were observed
394 in ROS levels (Maisanaba et al., 2013a). This might be due because GSH have many biological
395 functions apart from ROS scavenging such as signal transduction, gene expression and
396 apoptosis, etc. (Sies 1999). Moreover, Sharma et al. (2010) also reported that Closite®Na^+ and
397 Closite®30B did not induce ROS production in Caco-2, in agreement with our results for those
398 clays (Maisanaba et al., 2013a) and although there was no change in ROS levels they observed
399 genotoxic effects. By contrast, Lordan et al. (2011) reported that Cloisite®Na^+ induced
400 intracellular ROS formation, whereas, the effect of Cloisite®93A in the generation of ROS was
401 less prominent. Furthermore, Baek et al. (2012) evaluated the ROS production in INT-407 cells
402 exposed to MMT, obtaining significant levels of ROS at the highest concentration (1000 µg/ mL)
403 at all three timepoints assayed (24, 48 and 72h).
404
405 Clay 2 did not induce leakage of IL-6, biomarker of an inflammatory response, in any of the cell
406 lines. MMT, on the other hand, has been reported to rapidly lyses neutrophils and erythrocytes in
407 vitro. Furthermore, it can stimulate chemiluminescence, the neutrophil oxidative metabolic burst
408 (Dougherty et al., 1985).
409
410 In relation to genotoxicity, Clay 2 induced DNA strand breaks in both cell lines Moreover, Clay 2
411 induced time-dependent increases of DNA strand breaks in HepG2 cells. Similar results were
412 also found by Maisanaba et al., (2013a) for Cloisite30®B in HepG2 cells. Other authors evaluated
413 the genotoxicity of other commercial clays in similar cell lines. Sharma et al. (2010), showed no
414 DNA damage in Caco-2 exposed to unfiltered and filtered Cloisite®Na^+ samples. Nevertheless,
415 DNA damage was observed in the intestinal cell line Caco-2 exposed to Cloisite®30B from 113
416 µg/mL after 24h. By contrast, the results obtained by Maisanaba et al., (2013c) with the intestinal

417 cell line exposed to Cloisite®30B, did not coincide with the aforementioned research group, and
418 this can be related with the lower concentrations tested in this case (10, 20 and 40 µg/mL).
419 Maisanaba et al. (2013a), however, showed differences with respect to the control at the highest
420 concentration assayed (88 µg/mL) in HepG2 after 48 h. In this case, it is difficult to compare
421 because Sharma et al. (2010) only presented results after 24h, and at this concentration DNA
422 damage in the intestinal cell line was not observed.
423 Sharma et al. (2010), also indicated that the modifiers used to modify the clay contributed to the
424 genotoxic effects, and these effects were not due to oxidative damage. In this sense, the absence
425 of ROS obtained in this work after exposure to Clay 2 coincides with the Sharma´s hypothesis.
426 On the other hand, Li et al. (2010) observed no genotoxic results in the comet assay with CHO
427 cells exposed up to 1000µg/mL nanosilicate platelets derived from MMT.
428 Regarding to in vivo toxicity data, there is no information about the three organoclays selected in
429 the present study, but Li et al. (2010) did not reported acute oral toxicity of MMT in rats exposed
430 to a single dose of 5700 mg/kg. Also Baek et al. (2012) did not find any remarkable toxicity in
431 mice orally exposed to a single dose of 1000 mg/kg MTT.
432
433 In summary, our results showed that at the concentrations assayed Cloisite®20A and Clay 1 did
434 not induce cytotoxicity. Clay 2, however, showed cytotoxic and genotoxic effects, as well as an
435 alteration in GSH content. Therefore, a case by case toxicological assessment is required, as the
436 modifier has a role in the toxicity observed. Moreover, a different sensitivity was observed
437 depending on the cell line employed.
438 More studies are required to elucidate the risks of these clays for human health as their use is
439 going to increase widely in the near future.
440
441

442 **Acknowledgements**

443 The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de
444 Andalucía (AGR5969) for the financial support for this study, and the Cell Culture Service and
445 Microscopy Service of Centro de Investigación, Tecnología e Innovación de la Universidad de
446 Sevilla (CITIUS) for providing technical assistance.

447

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569 **Table captions**

570 Table 1. EC50 values (µg/mL) obtained after 24 and 48h exposure to Clay 2 in both cell lines.

571

572 **Figure captions**

573 Figure 1. TGA results for Cloisite®Na^+ (round) and Cloisite®20A (square).

574

575 Figure 2. FTIR results for Cloisite®Na^+ (up) and Cloisite®20A (down).

576

577 Figure 3. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
578 HepG2 (b,d) cells after 24h and 48h of exposure to 0-8 µg/mL Clay 1. Results from 3 independent
579 experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences
580 were considered significant from $p<0.05$ (*).

581

582 Figure 4. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
583 HepG2 (b,d) cells after 24h and 48h of exposure to 0-125 µg/mL Clay 2. Results from 3
584 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d.
585 Differences were considered significant from $p<0.05$ (*).

586

587 Figure 5. MTS tetrazolium salt reduction (a,b) and total protein content (c,d) of Caco-2 (a,c) and
588 HepG2 (b,d) cells after 24h and 48h of exposure to 0-62.5 µg/mL Cloisite®20A. Results from 3
589 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d.
590 Differences were considered significant from $p<0.05$ (*).

591

592 Figure 6. ROS content (a) in Caco-2 cells after 24 and 48h of exposure to 8.5, 17, or 34 µg/mL
593 Clay 2. ROS content (b) in HepG2 cells after 24 and 48h of exposure to 22, 44, or 88 µg/mL Clay

2. Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d.

Figure 7. GSH content (a) in Caco-2 cells after 24 and 48h of exposure to 8.5, 17, or 34 µg/mL Clay 2. GSH content (b) in HepG2 cells after 24 and 48h of exposure to 22, 44, or 88 µg/mL Clay 2. Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d. ** significantly different from control ($p < 0.01$).

Figure 8. Comet assay results (a) of Caco-2 and (b) HepG2 cells after 24 and 48h of exposure to 15.65, 31.25, or 62.5 µg/mL Cloisite®20A. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$).

Figure 9. Comet assay results (a) of Caco-2 and (b) HepG2 cells after 24 and 48h of exposure to 2, 4 or 8 µg/mL Clay 1. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$).

Figure 10. Comet assay results (a) of Caco-2 cells after 24 and 48h of exposure to 8.5, 17, or 34 µg/mL Clay 2. Comet assay results (b) of HepG2 cells after 24 and 48h of exposure to 22, 44 and 88µg/mL. Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. * significantly different from control ($p < 0.05$). ** significantly different from control ($p \leq 0.01$).

Table 1

Click here to download Table: Table 1.docx

Table 1

Cell line	Exposure time	EC50 (µg/mL)	
		MTS	PC
Caco-2	24h	55±6	34±3
	48h	26±3	18±2
HepG2	24h	88±4	93±5
	48h	51±3	74±3

Figure 1

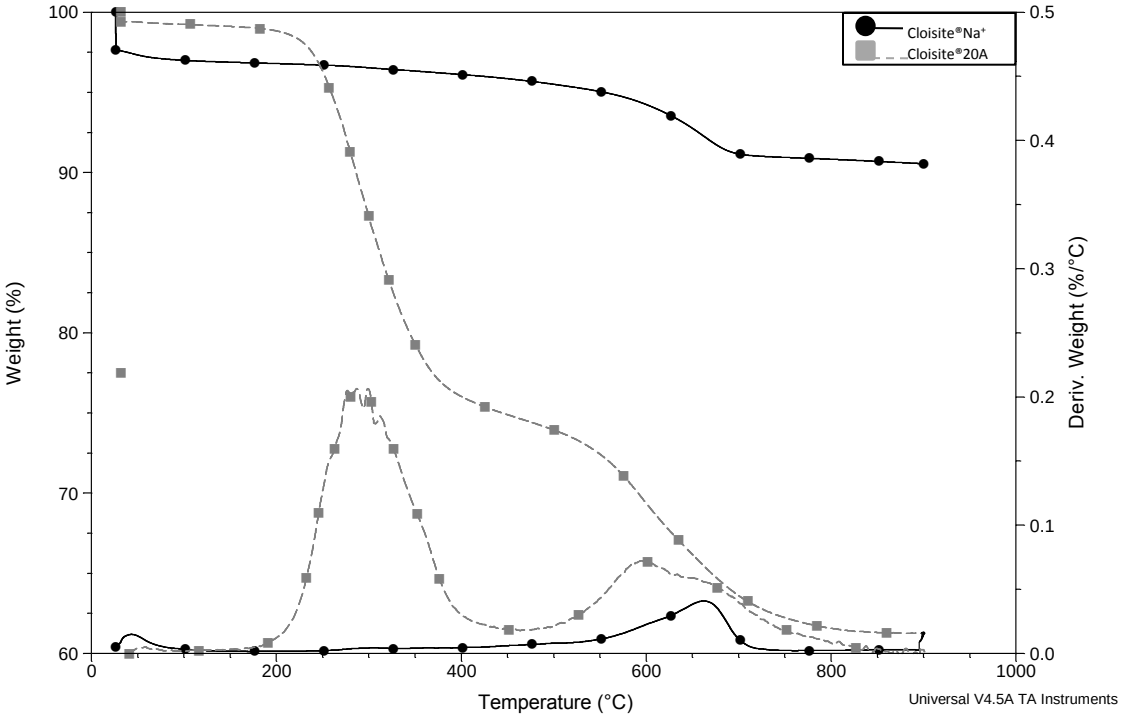

Cloisite®Na+
Cloisite®20A
Weight (%)
Deriv. Weight (%/°C)
Temperature (°C)
Universal V4.5A TA Instruments

Figure 2

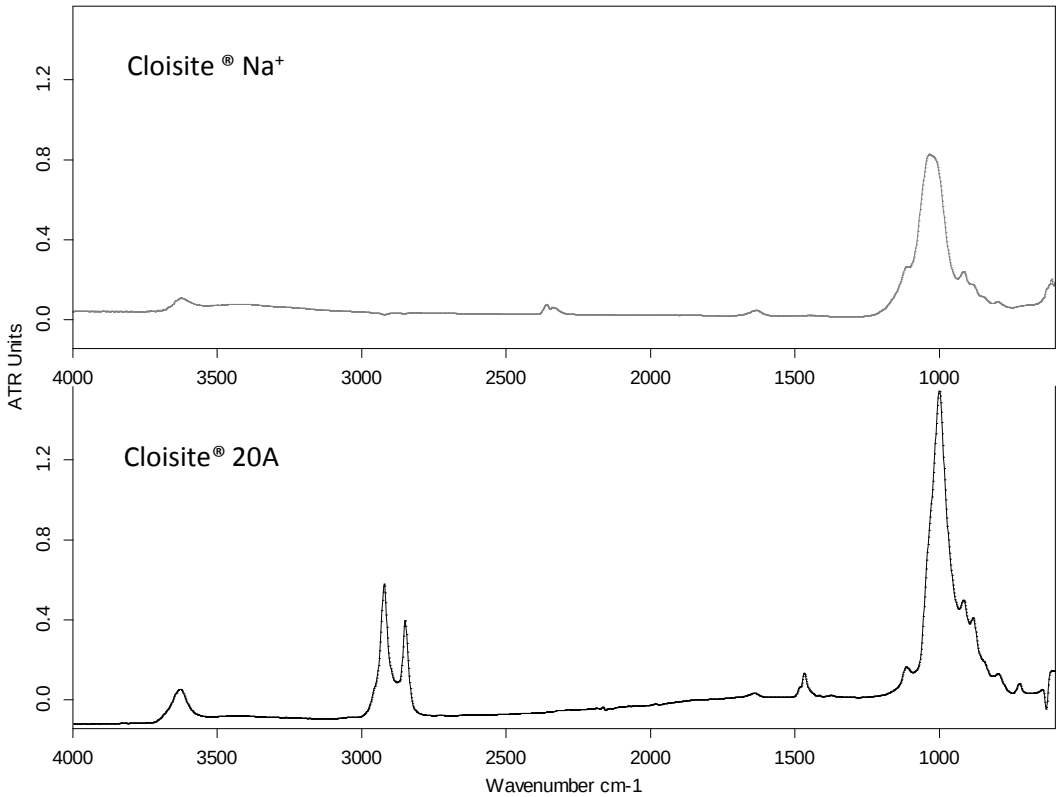
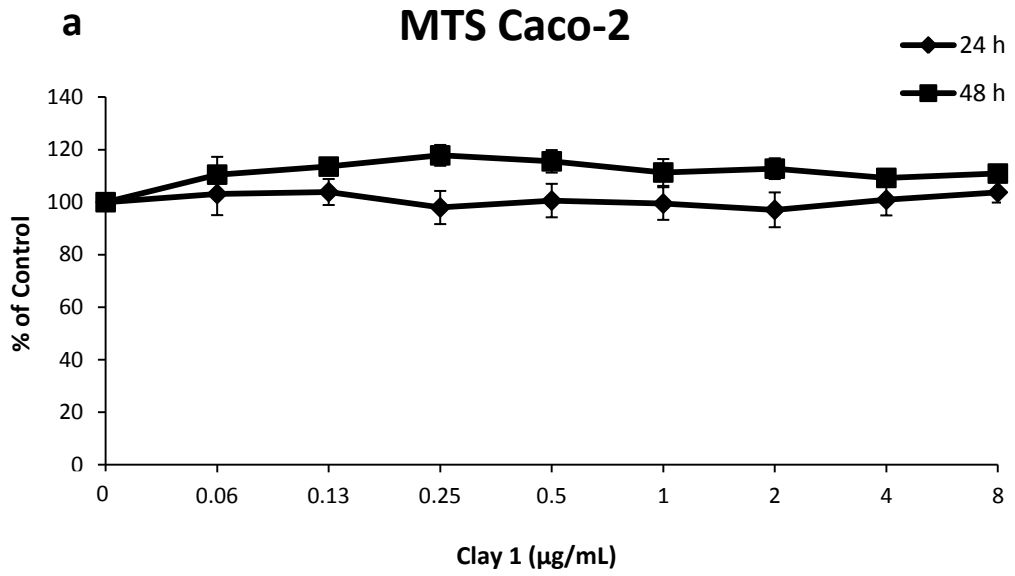
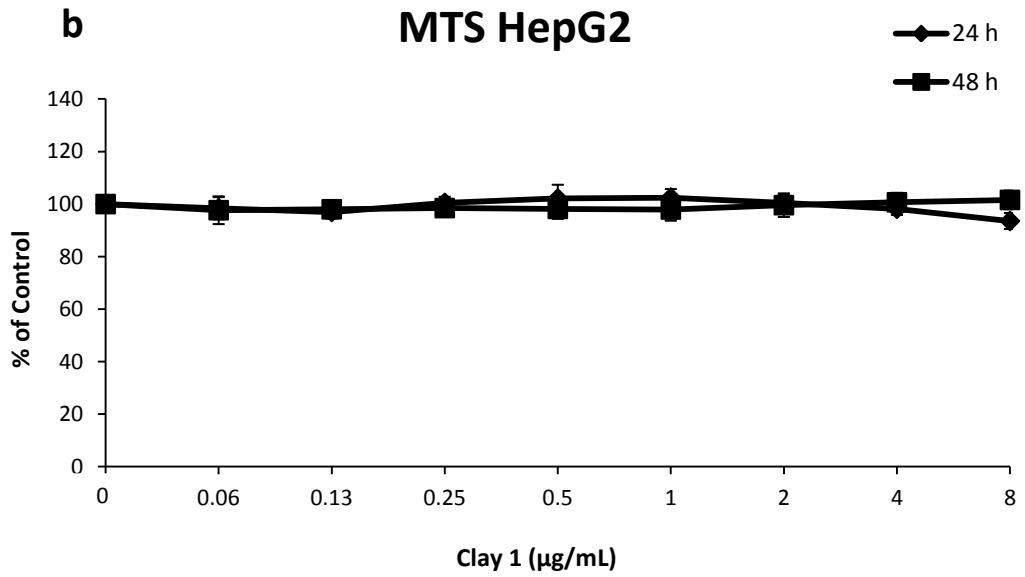
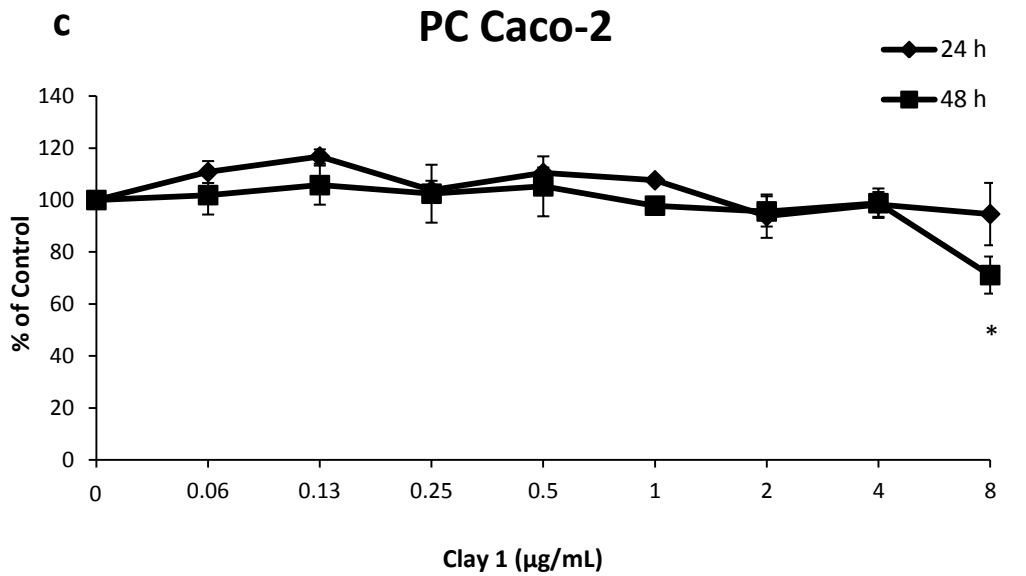

Cloisite ® Na+
Cloisite® 20A
ATR Units
0.0
0.4
0.8
1.2
4000
3500
3000
2500
2000
1500
1000
Wavenumber cm-1

Figure 3


a
MTS Caco-2
24 h
48 h
% of Control
0
20
40
60
80
100
120
140
0
0.06
0.13
0.25
0.5
1
2
4
8
Clay 1 (µg/mL)


b
MTS HepG2
24 h
48 h
% of Control
0
20
40
60
80
100
120
140
0
0.06
0.13
0.25
0.5
1
2
4
8
Clay 1 (µg/mL)


c
PC Caco-2
24 h
48 h
% of Control
0
20
40
60
80
100
120
140
0
0.06
0.13
0.25
0.5
1
2
4
8
*
Clay 1 (µg/mL)

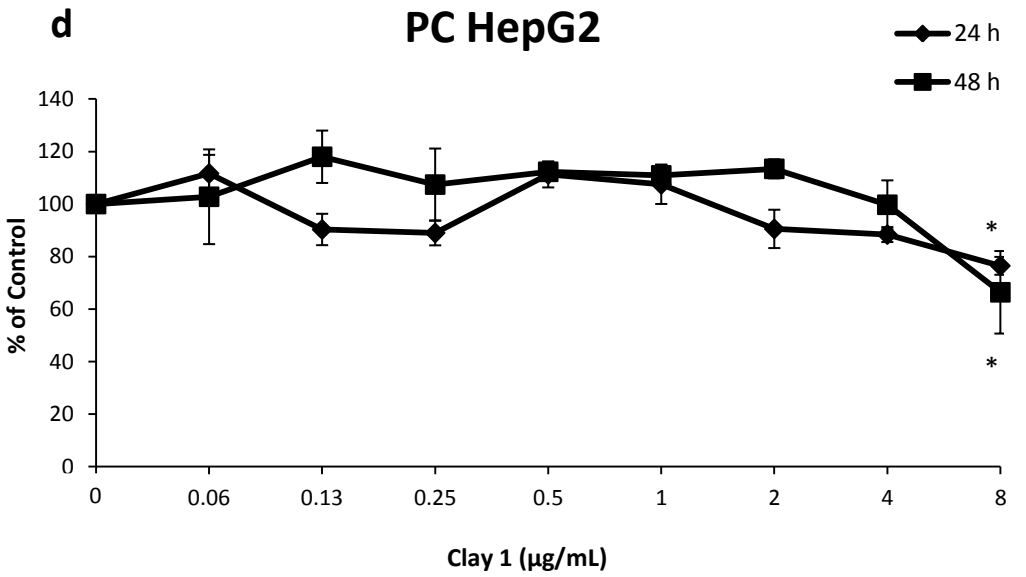
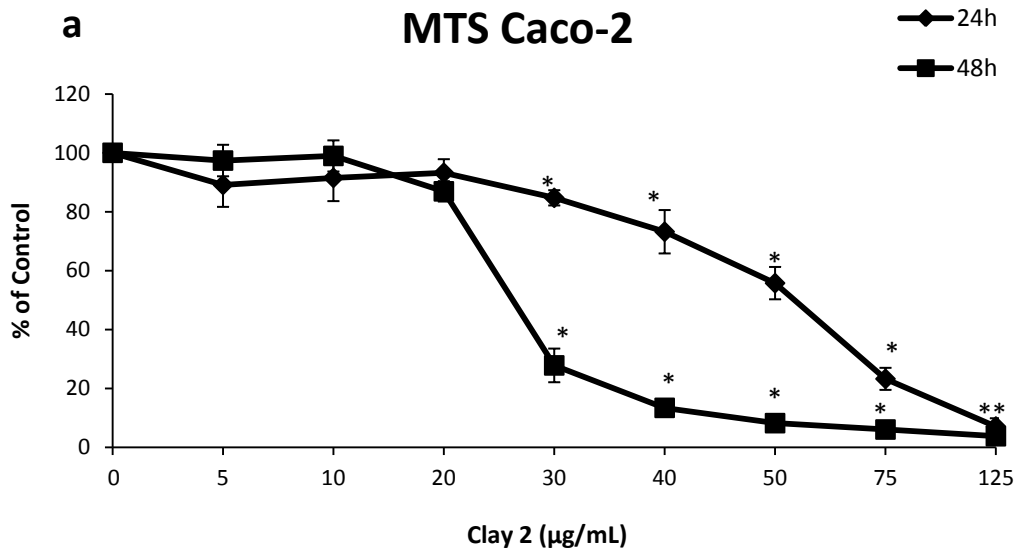
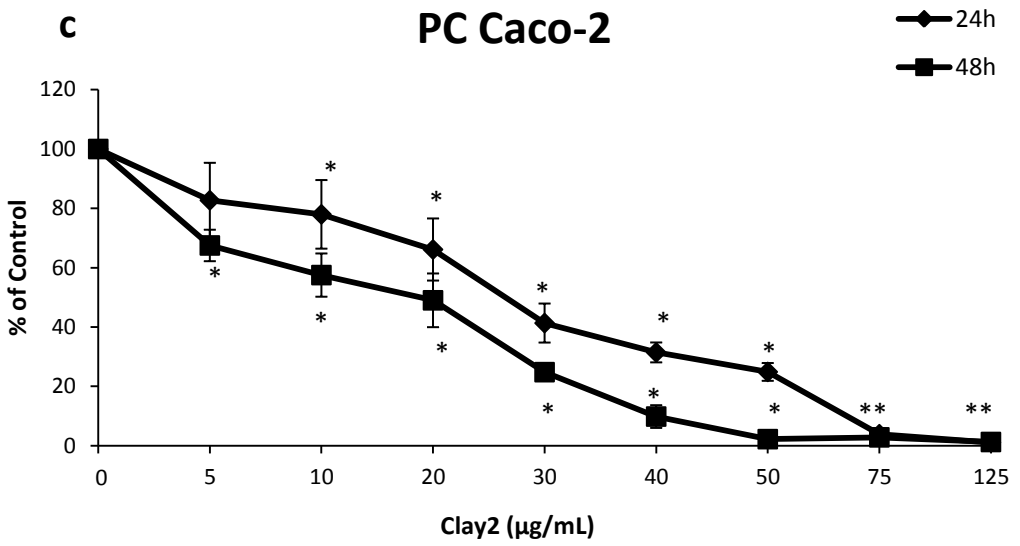
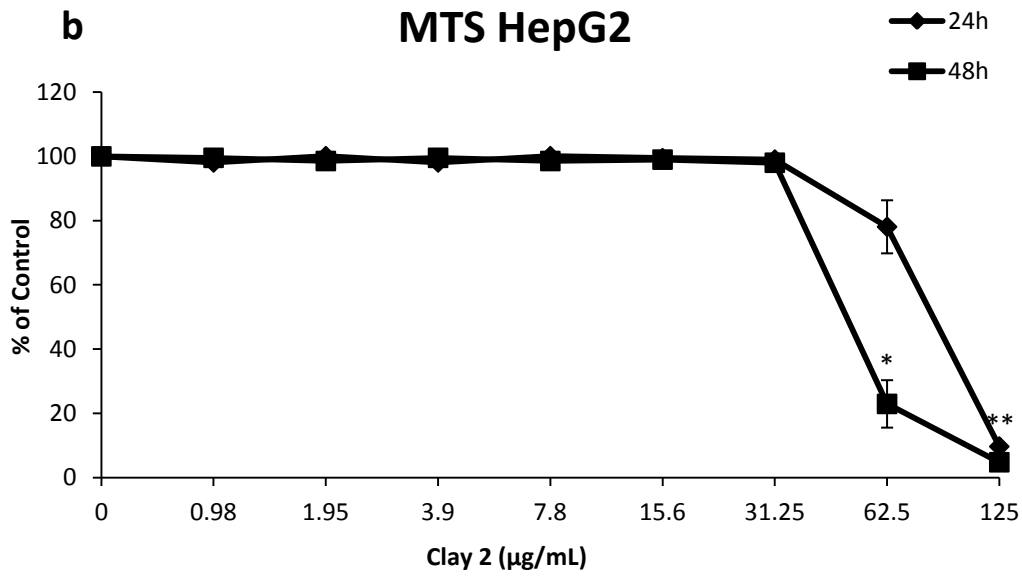

d
PC HepG2
24 h
48 h
% of Control
0
20
40
60
80
100
120
140
0
0.06
0.13
0.25
0.5
1
2
4
8
*
*
Clay 1 (µg/mL)

Figure 4


a
MTS Caco-2
24h
48h
% of Control
Clay 2 (µg/mL)


c
PC Caco-2
24h
48h
% of Control
Clay2 (µg/mL)


b
MTS HepG2
24h
48h
% of Control
Clay 2 (µg/mL)

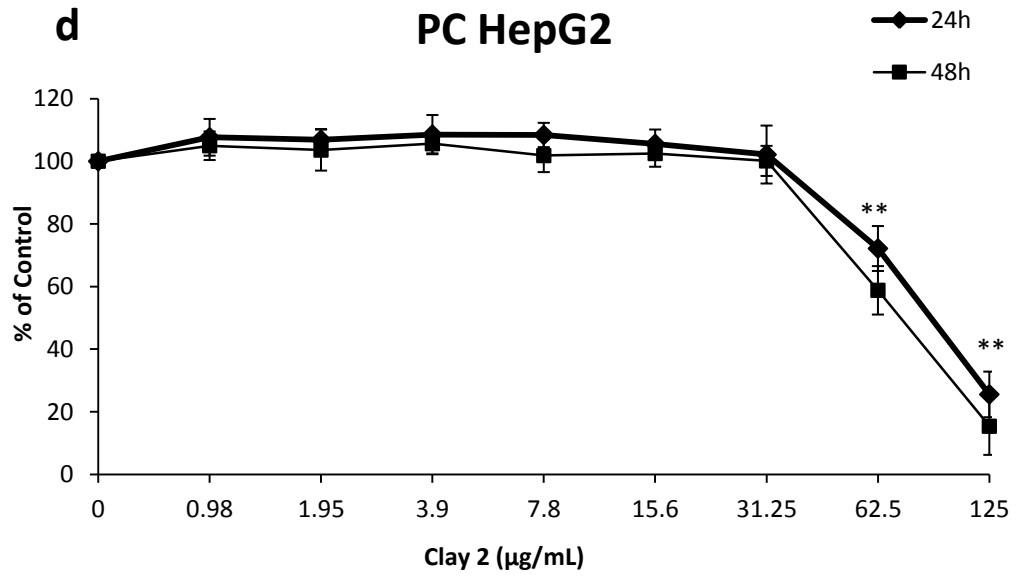
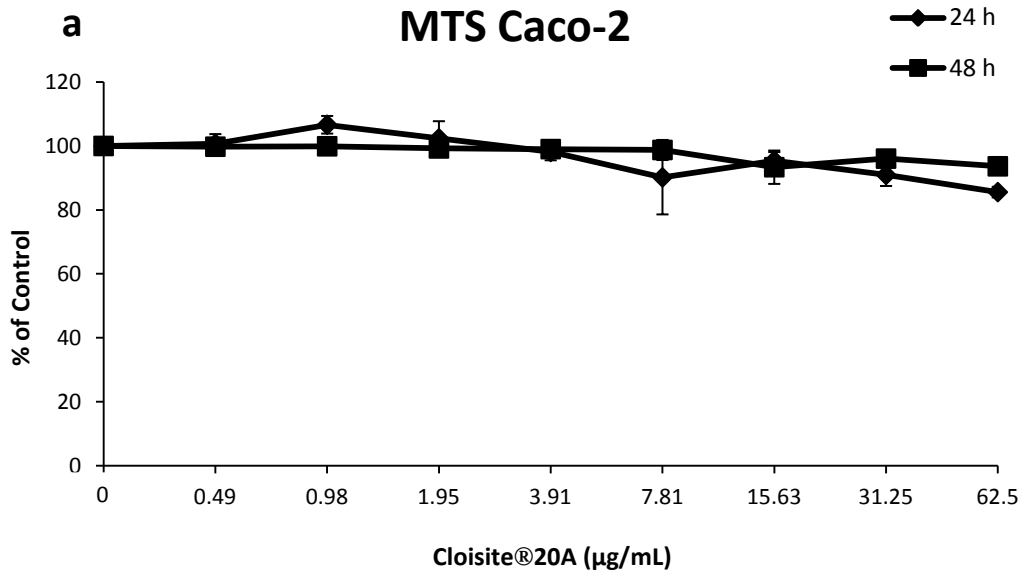
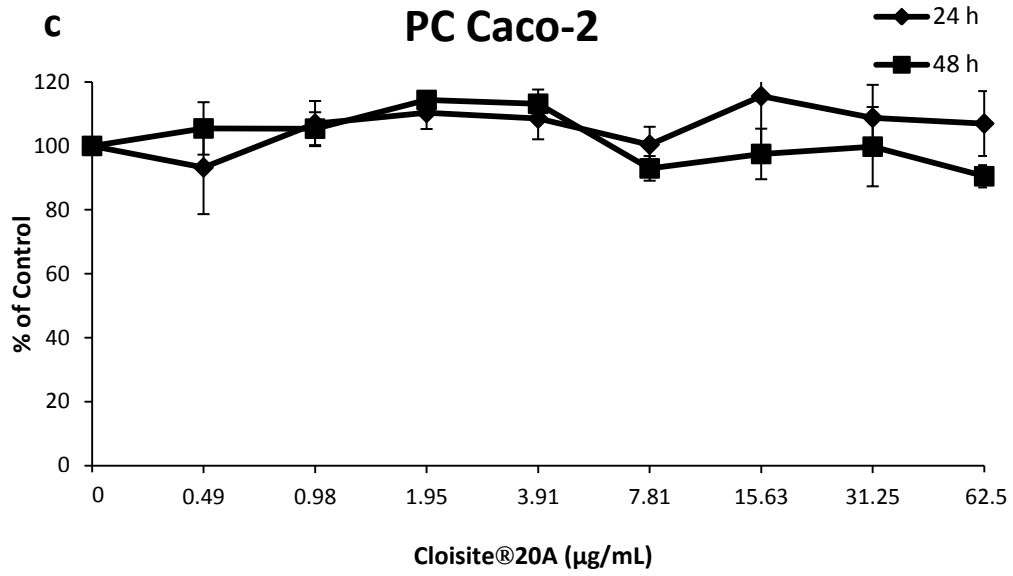
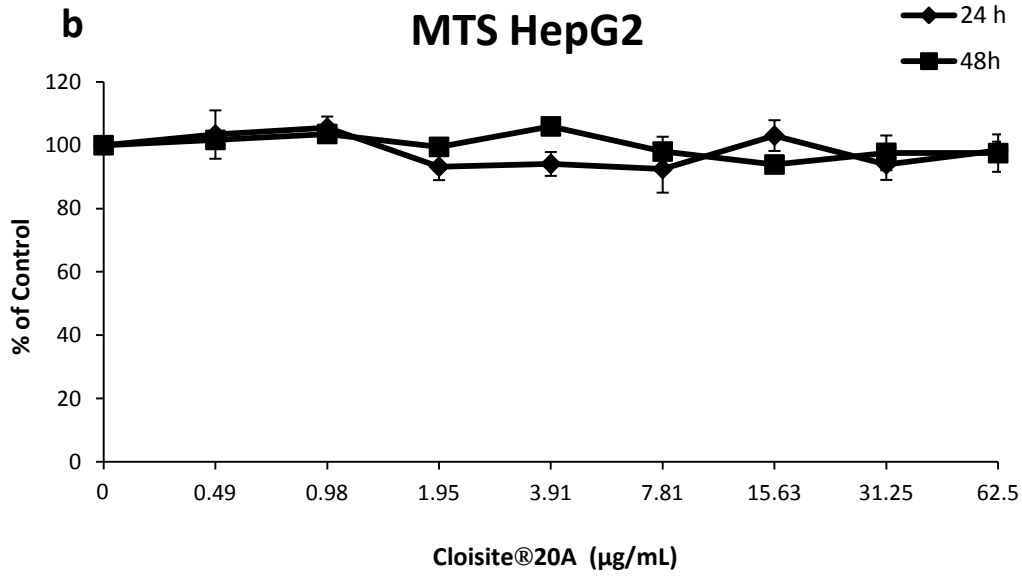

d
PC HepG2
24h
48h
% of Control
Clay 2 (µg/mL)

Figure 5


a
MTS Caco-2
24 h
48 h
120
100
80
60
40
20
0
% of Control
0
0.49
0.98
1.95
3.91
7.81
15.63
31.25
62.5
Cloisite®20A (µg/mL)


c
PC Caco-2
24 h
48 h
120
100
80
60
40
20
0
% of Control
0
0.49
0.98
1.95
3.91
7.81
15.63
31.25
62.5
Cloisite®20A (µg/mL)


b
MTS HepG2
24 h
48h
120
100
80
60
40
20
0
% of Control
0
0.49
0.98
1.95
3.91
7.81
15.63
31.25
62.5
Cloisite®20A (µg/mL)

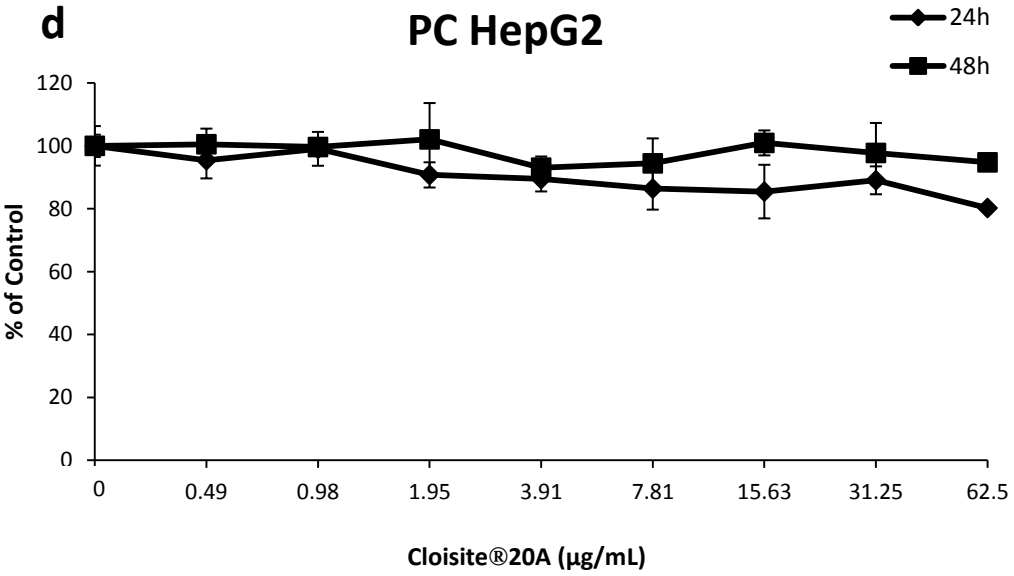
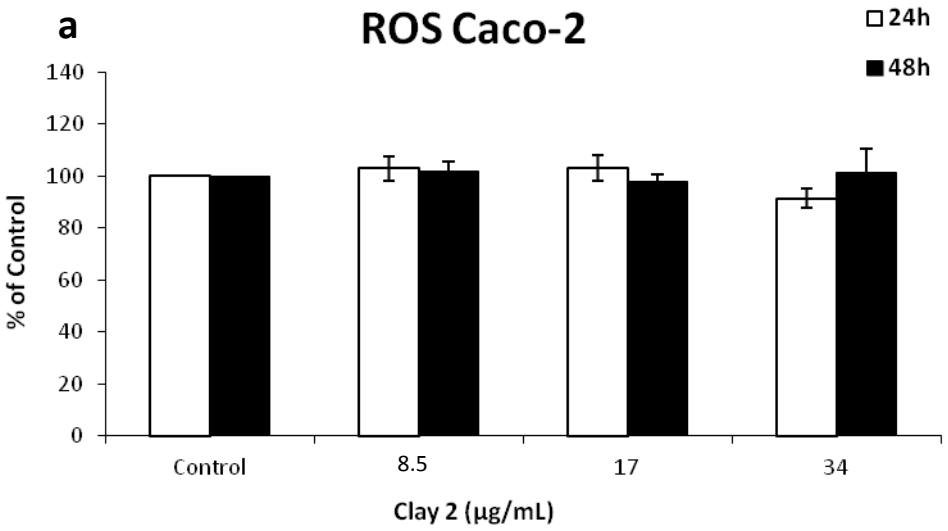

d
PC HepG2
24h
48h
120
100
80
60
40
20
0
% of Control
0
0.49
0.98
1.95
3.91
7.81
15.63
31.25
62.5
Cloisite®20A (µg/mL)

Figure 6


a
ROS Caco-2
24h
48h
140
120
100
80
60
40
20
0
% of Control
Control
8.5
17
34
Clay 2 (µg/mL)

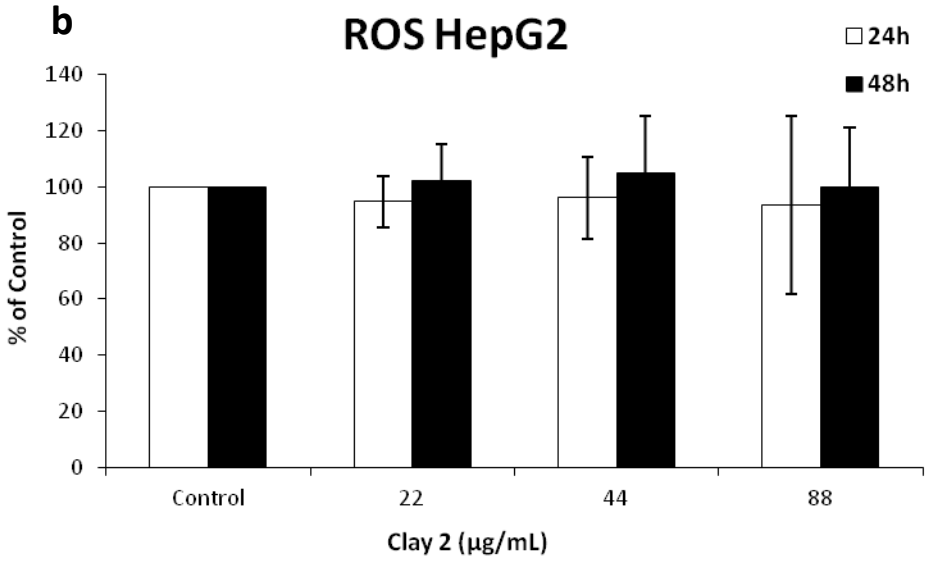
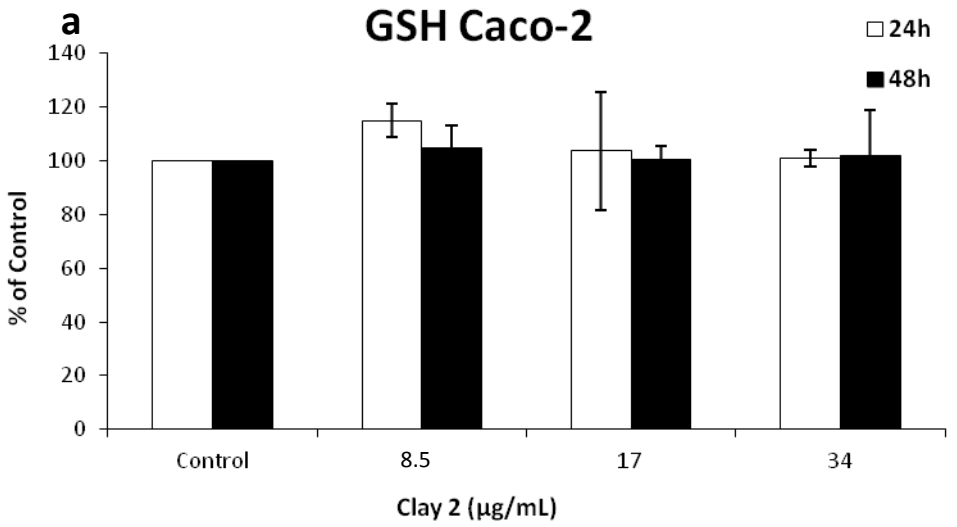

b
ROS HepG2
24h
48h
140
120
100
80
60
40
20
0
% of Control
Control
22
44
88
Clay 2 (µg/mL)

Figure 7


a
GSH Caco-2
24h
48h
% of Control
140
120
100
80
60
40
20
0
Control
8.5
17
34
Clay 2 (µg/mL)

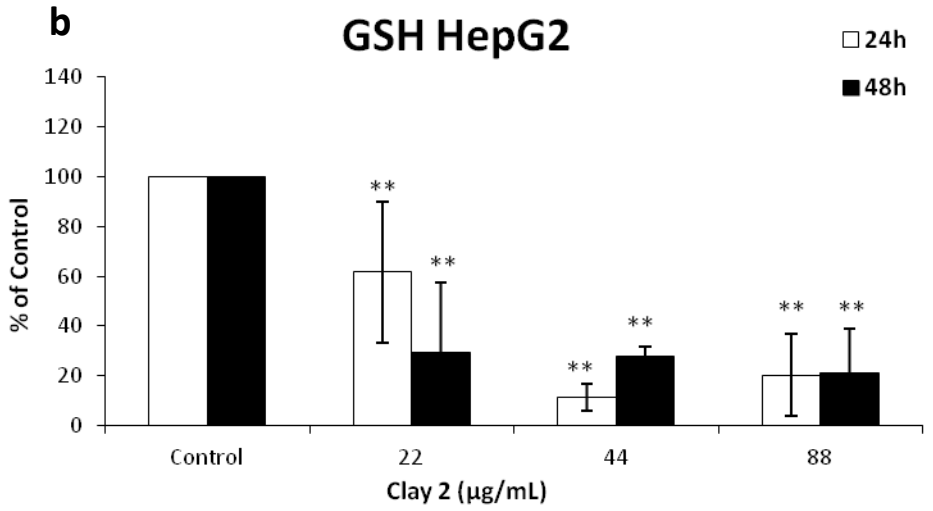
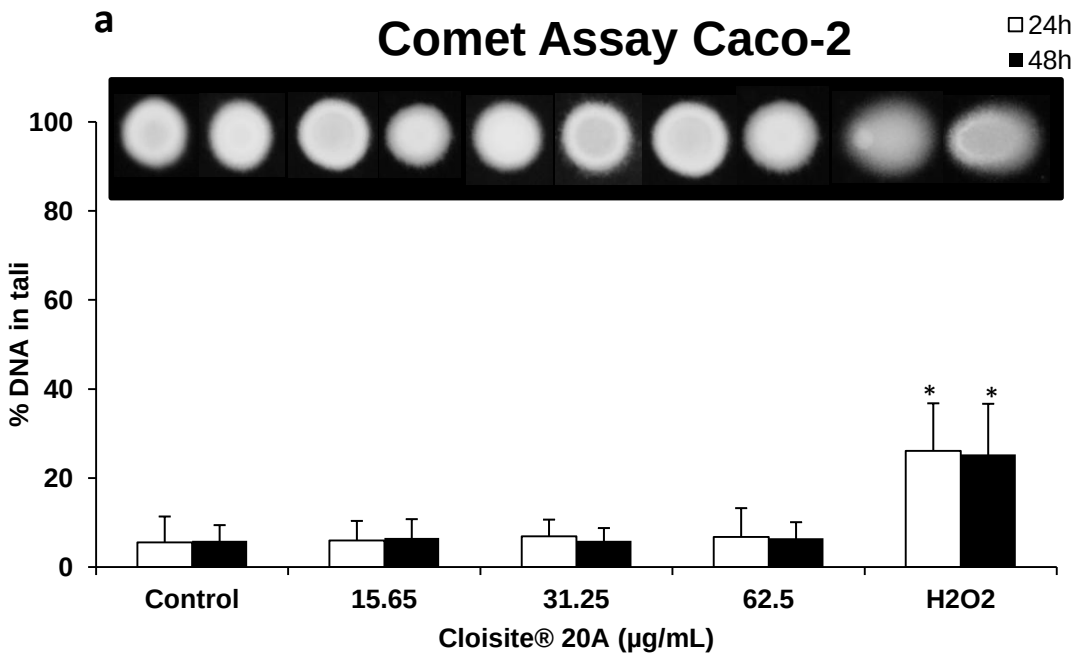

b
GSH HepG2
24h
48h
% of Control
140
120
100
80
60
40
20
0
**
Control
22
44
88
Clay 2 (µg/mL)

Figure 8


a
Comet Assay Caco-2
24h
48h
100
80
60
40
20
0
% DNA in tali
*
*
Control
15.65
31.25
62.5
H2O2
Cloisite® 20A (µg/mL)

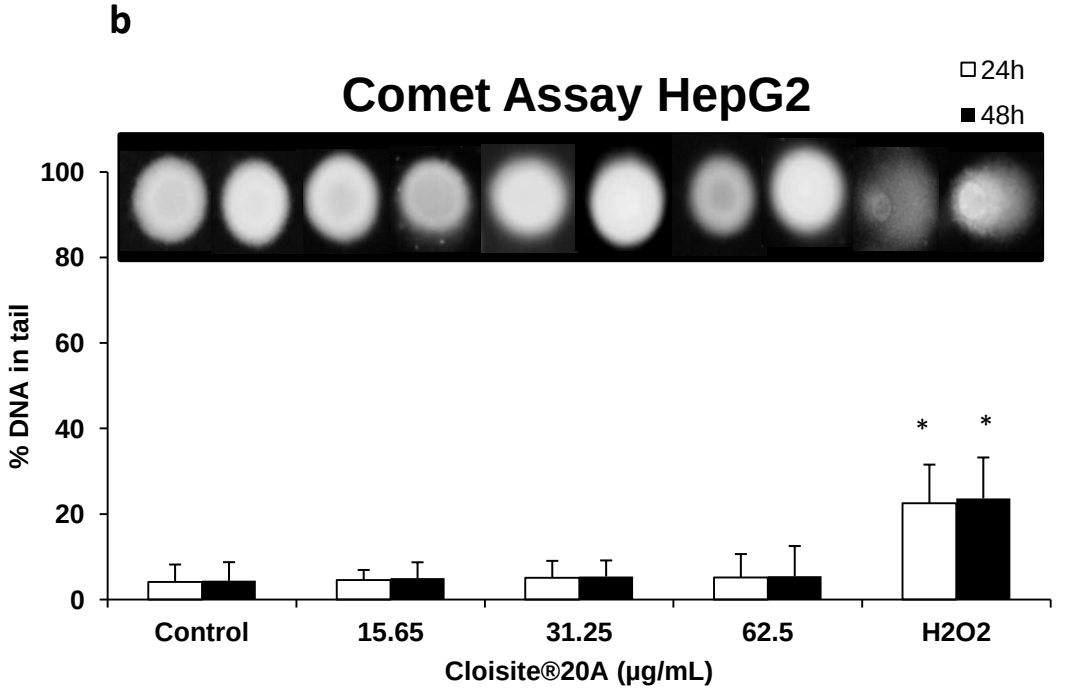

b
Comet Assay HepG2
24h
48h
100
80
60
40
20
0
% DNA in tail
*
*
Control
15.65
31.25
62.5
H2O2
Cloisite®20A (µg/mL)

Figure 9

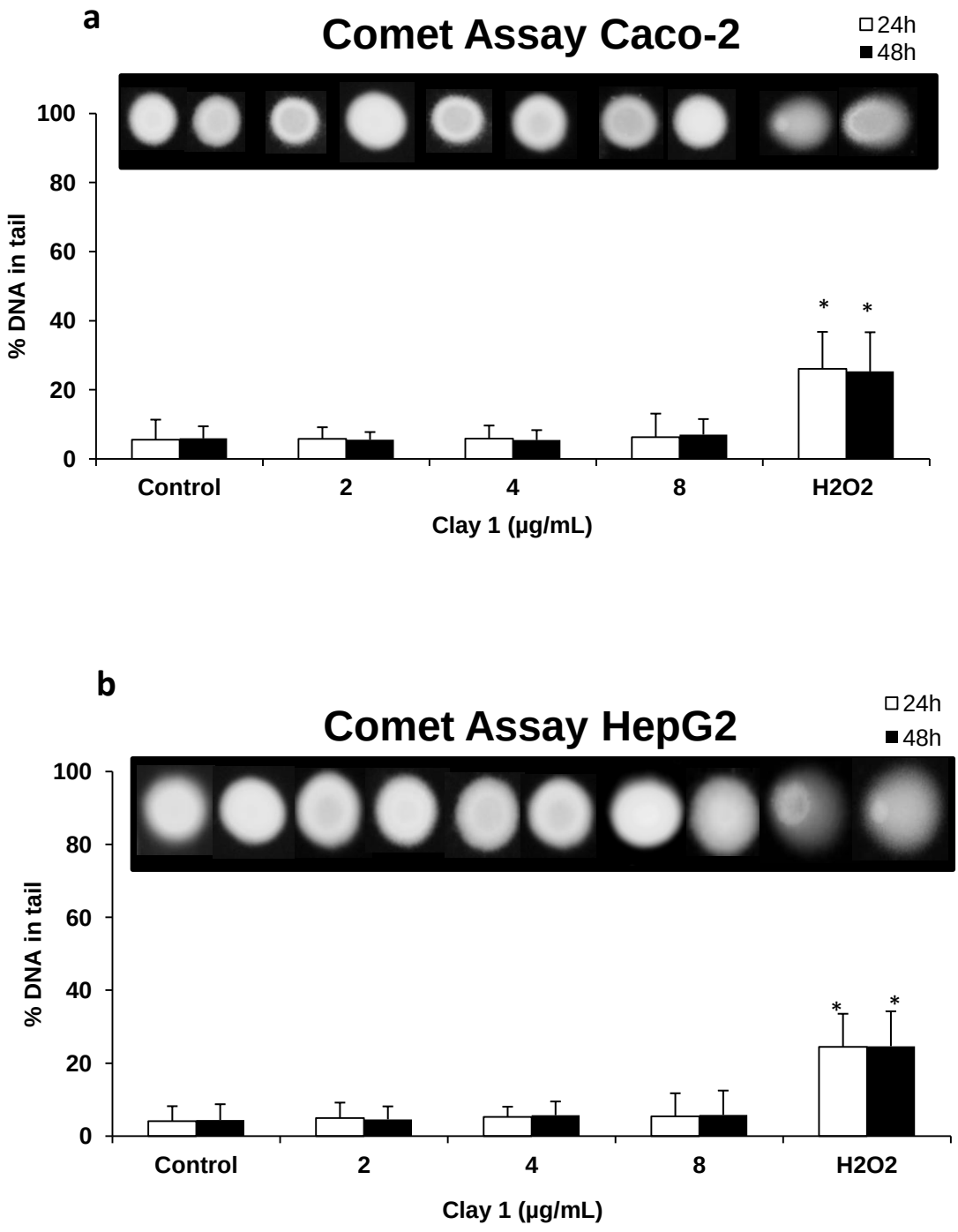
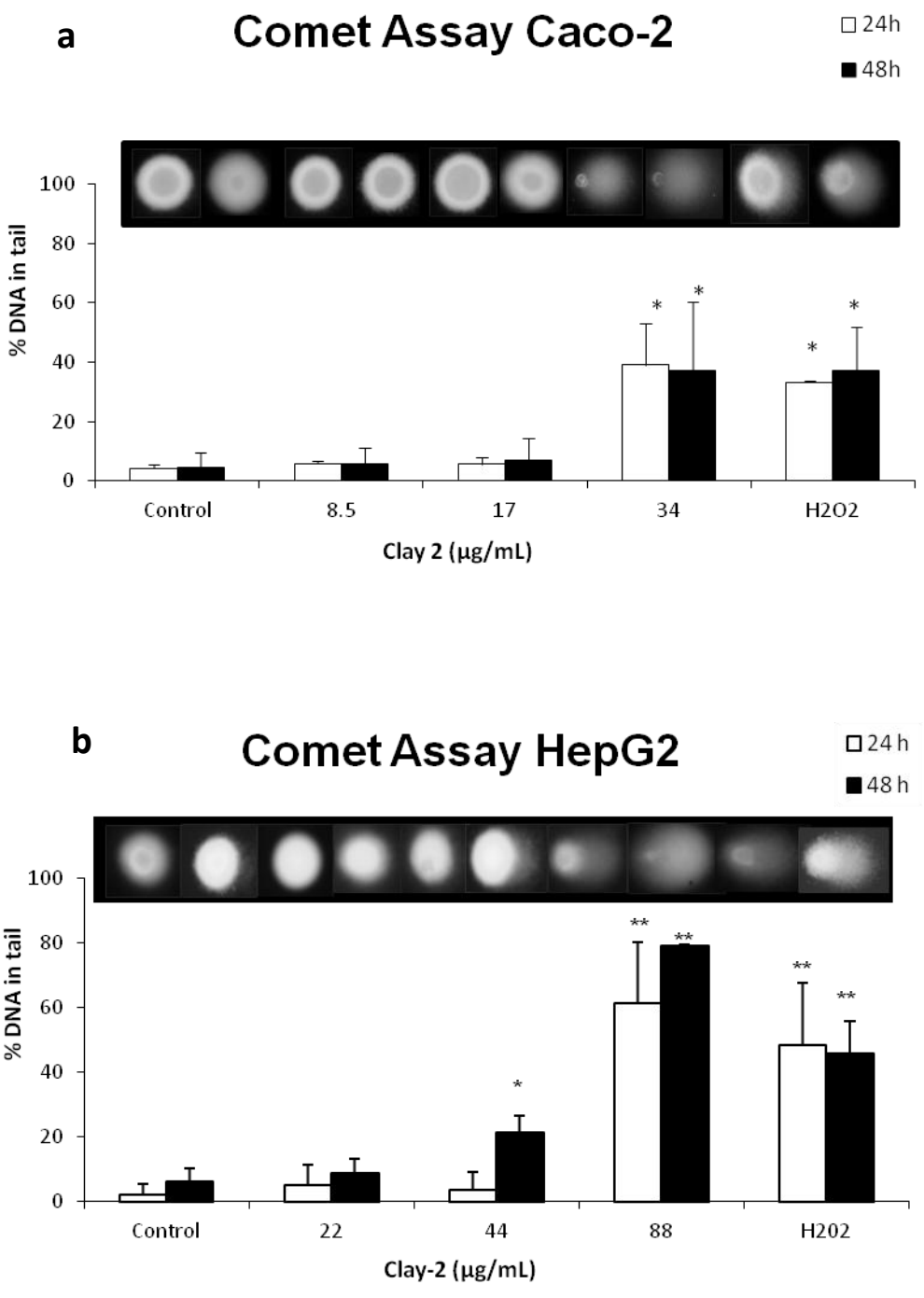

a
Comet Assay Caco-2
24h
48h
% DNA in tail
100
80
60
40
20
0
*
*
Control
2
4
8
H2O2
Clay 1 (µg/mL)
b
Comet Assay HepG2
24h
48h
% DNA in tail
100
80
60
40
20
0
*
*
Control
2
4
8
H2O2
Clay 1 (µg/mL)

Figure 10


a
Comet Assay Caco-2
24h
48h
% DNA in tail
100
80
60
40
20
0
Control
8.5
17
34
H2O2
Clay 2 (µg/mL)
b
Comet Assay HepG2
24 h
48 h
% DNA in tail
100
80
60
40
20
0
Control
22
44
88
H202
Clay-2 (µg/mL)

CAPÍTULO 8 / CHAPTER 8

Sara Maisanaba, Ana I. Prieto, Silvia Pichardo, María Jordá-Beneyto, Susana Aucejo, Ángeles Jos

CYTOTOXICITY AND MUTAGENICITY ASSESSMENT OF ORGANOMODIFIED CLAYS POTENTIALLY USED IN FOOD PACKAGING

Toxicology In Vitro 20, 1222-1230, ***2015***

Elsevier Editorial System(tm) for Toxicology in Vitro
Manuscript Draft

Manuscript Number:

Title: Cytotoxicity and mutagenicity assessment of organomodified clays potentially used in food packaging

Article Type: SI:The ESTIV 2014 proceeding

Keywords: clay minerals, cytotoxicity; mutagenicity; Ames test; HUVEC

Corresponding Author: Dr. Silvia Pichardo, PhD

Corresponding Author's Institution: Universidad de Sevilla

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Ana I Prieto; Silvia Pichardo, PhD; Maria Jordá-Beneyto; Susana Aucejo; Angeles Jos

Abstract: Modern food packaging has made great advances as result of global trends and consumer preferences, which are oriented to obtain improved food quality and safety. In this regard, clay minerals, and mainly Montmorillonite (Mt) are attracting considerable interest in food packaging because of the improvements developed in mechanical and barrier properties.. Hence, the present work aim to assess the toxicity of four Montmorillonite-based clay minerals, an unmodified clay, Cloisite®Na+ (CNa+), and three modified Mt clays: Cloisite®30B (C30B), a commercial clay, and Clay1 and Clay2, two novel modified organoclays developed by the Packaging, Transport, & Logistics Research Institute (ITENE). First, the cytotoxic effects were studied in the human endothelial cell line (HUVEC). In addition, the potential mutagenicity of the clays was evaluated by the Ames test. Clay1 did not induce any cytotoxic effects in HUVE cells, although it exhibited potential mutagenicity in TA98 S. thyphimurium strain. In contrast, Clay2 produced cytotoxicity in endothelial cells but no mutagenicity was recorded. However, CNa+ was not cytotoxic neither mutagenic. And finally, C30B showed positive results in both assays. Therefore, results showed that clay minerals have a different toxicity profile and a case by case toxicity evaluation is required.

Silvia Pichardo
Area of Toxicology. Faculty of Pharmacy. University of Seville
C/Profesor García González 2, 41012 Seville, Spain
Tel.: +34-954556762; fax: 34-954556422.
E-mail address: spichardo@us.es

23rd August, 2014

Dear Editor,

We would be very grateful if you consider the manuscript entitled **"Cytotoxicity and mutagenicity assessment of organomodified clays potentially used in food packaging"** for its publication in "Toxicology in Vitro".

The work included in the present manuscript has been presented in the Conference ESTIV 2014 by Ms Sara Maisanaba with the following titles:

- "CYTOTOXICITY OF UNMODIFIED AND MODIFIED CLAYS IN AN ENDOTHELIAL CELL LINE"

- "MUTAGENICITY AND GENOTOXICITY IN A TARGET CELL LINE OF THE MODIFIED CLAYS: CLAY1 AND CLAY2"

Unfortunately, the manuscript was not ready to be sent as proceedings in this Conference. But I hope this time it could be considered to be published in your journal.

To the extent of our knowledge this is the first work dealing cytotoxic effects of four montmorillonite-based clay minerals, an unmodified clay, Cloisite®Na+, and three modified Mt clays: Cloisite®30B, a commercial clay, and Clay1 and Clay2, two novel modified organoclays, on the human cell line HUVE cells; as well as studying their mutagenic potential in order to assess their safety in regard to be used in food packaging.

The authors declare that there are no conflicts of interest.

I am looking forward to receiving a positive answer from you.

Sincerely,

Silvia Pichardo

Cytotoxicity and mutagenicity assessment of organomodified clays potentially used in food packaging

Sara Maisanaba[a], Ana I Prieto [a], Silvia Pichardo[a*], María Jordá-Beneyto[b], Susana Aucejo[b], Ángeles Jos[a]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[b] Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

[*]To whom correspondence should be addressed at:

Silvia Pichardo Sánchez

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: spichardo@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Modern food packaging has made great advances as result of global trends and consumer preferences, which are oriented to obtain improved food quality and safety. In this regard, clay minerals, and mainly Montmorillonite (Mt) are attracting considerable interest in food packaging because of the improvements developed in mechanical and barrier properties. Hence, the present work aim to assess the toxicity of four Montmorillonite-based clay minerals, an unmodified clay, Cloisite®Na+ (CNa+), and three modified Mt clays: Cloisite®30B (C30B), a commercial clay, and Clay1 and Clay2, two novel modified organoclays developed by the Packaging, Transport, & Logistics Research Institute (ITENE). First, the cytotoxic effects were studied in the human endothelial cell line (HUVEC). In addition, the potential mutagenicity of the clays was evaluated by the Ames test. Clay1 did not induce any cytotoxic effects in HUVE cells, although it exhibited potential mutagenicity in TA98 *S. thyphimurium* strain. In contrast, Clay2 produced cytotoxicity in endothelial cells but no mutagenicity was recorded. However, CNa+ was not cytotoxic neither mutagenic. And finally, C30B showed positive results in both assays. Therefore, results showed that clay minerals have a different toxicity profile and a case by case toxicity evaluation is required.

Keywords: clay minerals, cytotoxicity; mutagenicity; Ames test; HUVEC.

Introduction

Clay minerals have many potential applications due to their specific properties (Sharma et al., 2010). Montmorillonite (Mt) is a bio-inspired layered clay mineral widely used in various science fields due to their good swelling capacity, high specific surface area, good cation-exchange capacity, high platelet aspect ratio, strong adsorptive power, high structural stability, chemical inertia, strong capacity to form stable suspensions and ease with which their surface can be modified (Hu et al., 2007; Mallakpour and Dinari, 2011; Baek et al., 2012). Layered clays are of particular interest due to the successful incorporation into composite materials and the advantageous properties that reinforced materials exhibit, which is essential when high barrier properties are needed, for example, in food packaging (Rhim and Ng, 2007). But for the successful incorporation of the clays to the polymer matrix their compatibility should be improved by means of surface modification (Bitinis et al., 2011). This is accomplished, among others methods, by cation exchange with organic cations leading to organoclays (Maisanaba et al., 2014a). Once the clays have been incorporated into the polymer they become into nanoclays. These are defined as natural layered structures, where the layers or platelets have sub-micrometer dimensions, except for their thickness, which is only about 1 nm (Hatzigrigoriou et al., 2011). These platelets force gases and other external agents to follow a tortuous path through the material, decreasing and slowing the contact with the product obtaining more resistant plastics (Pereira de Abreu et al., 2007). In this regard, the Technological Institute of Packaging, Transport and Logistic (ITENE) is developing modified clays based in Mt to be incorporated in food packaging. Clay1 was prepared with Mt modified with

hexadecyltrimethyl-ammonium bromide (HDTA) and Clay2 contained HDTA and acetylcholine chloride as modifiers (Jordá-Beneyto et al., 2014).

New approaches and standardized test procedures to study the impact of organoclays on living cells are urgently needed for the evaluation of potential hazards relating to human exposure to these substances (Sekhon, 2010). In this sense, in vitro assays could be useful as screening method to detect toxicity. Several authors have evaluated the potential toxicity of modified clay minerals destined to food packaging in target cell lines from intestinal (Houtman et al., 2014; Maisanaba et al., 2014; Sharma et al., 2010) or hepatic origin (Lordan et al., 2011; Maisanaba et al., 2013). However there are other potential targets for clay minerals, such as the vascular endothelial tissue, with no information of their effects on HUVEC permanent cell line.

Additionally, cytotoxic effects are frequently induced by high doses at acute exposure. Therefore, subtle cellular alterations that may arise at lower concentrations are overlooked (Singh et al., 2009). In this regards, genotoxicity studies should be included in the toxicity assessment of new substances that human could be routinely exposed to, such as clay minerals used in food packaging. Actually, European legislation in relation to the authorization of food contact materials includes genotoxicity tests among the toxicological assays required (European Commission, 2011) Therefore, the first step in the toxicological assessment of clay minerals intended to be used in food packaging should be the cytotoxic and genotoxic evaluation of the material as manufactured, this is, before being incorporated in the package.

Taking into account all this background, the present work aims to assess the toxicity of four Mt-based clays, an unmodified clay, Cloisite®Na+ (CNa+), and three modified Mt clays: Cloisite®30B (C30B), a commercial clay, and Clay1 and Clay2, two novel modified organoclays developed by ITENE. The toxicological assessment included the cytotoxicity in a human endothelial cell line (HUVEC) and the genotoxicity by the Ames test after 24 and 48 hours of exposure to the selected clay minerals.

Materials and Methods

Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

Clay minerals characterization

Purified sodium montmorillonite (Mt) (CNa+), with cationic exchange capacity (CEC) of 116 meq/100 g as well as the organically modified one (C30B) (modifier: methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium, concentration: 90 meq/100g clay) were purchased from Southern Clay Products (COMITEX, S.A. Spain).

Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) ($C_{19}H_{42}BrN$, 364.46 g/mol, 98%) was supplied from CymitQuimica S.L. (Spain), acetylcholine chloride (ACO) ($C_7H_{16}ClNO_2$, 181.66 g/mol, P99%) was provided by Sigma-Aldrich (Spain).

The non commercial organo-modified clays, Clay1 and Clay2, have been developed and characterized by ITENE based on a previous work (Jordá-Beneyto et al., 2014). Briefly, two different organo-modified clays were prepared by a cation-exchange method, which consists on a displacement of the sodium cations of CNa+ with the ammonium cations of the above mentioned salts, obtaining Clay1 and Clay2.

Clay minerals were characterized by termogravimetric analysis (TGA), Fourier Transform InfraRed (FTIR), X-ray diffraction (XRD) and Particle size Distribution (PSD) as described in Jordá-Beneyto et al. (2014).

Cell culture

HUVEC permanent cell line derived from a human vascular endothelium (ATCC No. CRL-1730) were maintained at 37º C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO2 incubator, NuAire®, Spain), in a medium consisting in F-12K (LGC Standards, Spain) supplemented with fetal bovine serum, heparin and endothelial cell growth supplement. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by exclusion of Trypan Blue. HUVECs were plated at density of 5 x 10^4 cells/ml to perform the experiments.

Cytotoxicity assays

The test concentrations of the clay minerals were determined individually in previous experiments in order to avoid interferences with the method of measurement. For this reason the absorbance of clay solutions (1000 µg/mL and serial half dilutions) were

measured at 0, 24 and 48 h. The highest concentrations selected were the ones that did not show statistical differences versus the control. Stock solutions were 1000 µg/mL for each clay. From the initial solution, serial dilutions in medium were prepared (0-125 µg/mL for CNa+; 0-250 µg/mL for C30B; 0-8 µg/mL for Clay1 and 0-125 µg/mL for Clay2). Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37ºC for 24 and 48 hours. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR), and tetrazolium salt reduction (MTS).

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage with independence of the toxic mechanism involved (Pichardo et al., 2007). PC was quantified *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the total cell number present in the culture. The culture medium was replaced by 200 µL NaOH 0.1N and after 2h incubation at 37ºC, 180 µL was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm (Infinite M200, Tecan, Austria).

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, NR in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of

viable cells. The NR can be extracted from lysosomes for quantitative measurement of cell viability and cytotoxicity of xenobiotics. Culture medium was replaced by 100 µL medium without serum containing 10 mg/mL NR. The 96-well plate with the NR-containing medium was returned to the incubator for another 3 h to allow the uptake of NR into the lysosomes of viable, intact cells. Thereafter, the medium was removed and cells were fixed for 1 min with a formaldehyde-$CaCl_2$ solution. By adding 0.2 mL of acetic acid-ethanol solution to the wells, the NR absorbed by the cells was extracted, brought into solution and quantified at 540 nm (Infinite M200, Tecan, Austria).

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS tetrazolium compound added to the medium is bioreduced by cells seeded in the 96-well plate into a colored formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm (Infinite M200, Tecan, Austria) after 2 hours of incubation in the dark.

Ames Test

The incorporation version of the Ames test was performed according to the recommendations of Maron & Ames (1983) and following the principles of OCDE guideline 471 (1997). Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used for the assay. Cultures of each

tester strain were prepared from their main strain plates, and used in late exponential growth phase. Each test substance was assessed in three independent experiments, each conducted in absence and presence of S9 metabolic activation system from rat liver (Aroclor 1254, Sigma, Spain), using triplicate plates for each test substance concentration. Five different concentrations of the clay minerals were tested. Briefly, 0.1 mL of test substance, 0.1mL of bacterial culture and 0.5mL of S9 mix, were added to 2 mL molten agar at 42° and poured onto Vogel Bonner-E minimal glucose agar plates. Plates were inverted and incubated 65-70 h at 37°C in the dark. Revertant colonies were counted and background lawn was inspected for signs of toxicity or compound precipitation. 2-Nitrofluorene (2-NF) (0.1 µg/plate) and sodium azide (NaN_3) (1 µg/plate) were selected as positive controls for assays performed without metabolic activation system. 2-Aminofluorene (2-AF) (20 µg/plate) was the positive control used in plates with S9 mix. MilliQ water (100µl/plate) and DMSO (10µl /plate) were selected as negative controls for this assay, being the solvents of clays solution and positive controls, respectively.

Calculations and statistical analysis

All experiments were performed at least three times and in duplicate per concentration. Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were

considered significant from P<0.05. All values passed the normality tests (Kolmogorov & Smirnov's test, and Bartlett´s test).

EC_{50} values (mean effective concentration, concentration that modified each biomarker by 50%, positive or negative, in comparison with appropriate untreated controls) were derived by linear regression in the concentration-response curves.

Results

Results of the cytotoxicity assays

HUVE cells exposed to CNa+ showed no significant changes in any of the endpoints assayed after 24 and 48 h of exposure (Figure 1).

On the other hand, when HUVE cells were exposed to C30B a concentration and time-dependent decrease was recorded in all endpoints. PC content was significantly reduced from the concentrations of 31.25 µg/mL C30B in cells exposed for 24 h and from the concentration of 15.63 µg/mL C30B after 48 h of exposure (Figure 2a). Moreover, significant differences were observed for the EC_{50} values in the PC content assay, being lower in the longest exposure time (64.3±9.7 µg/mL for 24 h and 28.3±11.2 µg/mL for 48 h). Similarly, NR uptake assay and MTS reduction indicated significant decreases in cell viability at the concentration 31.25 µg/mL at 24 h, and from 15.63 µg/mL in cells exposed for 48h (Figure 2b,c); although the level of statistical significance was higher in comparison to those obtained in PC content assay. The EC_{50} values for the NR uptake assay and MTS assay also evidenced a greater effect in cells

after a longer exposure time, being 49.7±10.0 and 42.3±9.5 µg/mL for 24h, respectively, and 21.4±11.4 and 24.4±10.4 µg/mL for 48h, respectively).

Clay1 did not induce any significant alterations in PC content and NR uptake assays in HUVE cells at any exposure and concentration used (Figure 3a,c). However, MTS reduction assay showed a significant decrease at the highest concentration assayed (8 µg/mL) after 48 h of exposure (Figure 3b).

HUVE cells exposed to Clay2 underwent a concentration and time-dependent decrease in all the endpoints assays. Significant reductions in cell viability measured by PC content were shown from the concentration of 15.63 µg/mL Clay2 in cells exposed for 24 and 48 h of exposure (Figure 4a). In addition, considering the EC_{50} values obtained, toxic effects were higher in the longest exposure, being 61.8±6.1 µg/mL for 24 h and 47.6±6.1 µg/mL for 48 h. NR uptake assay also indicated a reduction in cell viability. These decreases were significantly different from the control group at the concentration 15.63 µg/mL at 24 h, and from 7.8 µg/mL in cells exposed for 48h (Figure 4b). Moreover, marked differences were observed for the EC_{50} values in the NR uptake assay, being significantly lower after 24 h in comparison to 48 h (123.9±2.2 µg/mL for 24h and 23.1±9.1 µg/mL for 48h). Similarly, MTS reduction performed by cells exposed to Clay2 also decreased from 31.25 µg/mL at both exposure times (Figure 2c). However, the EC_{50} values for the MTS assay at both exposure times were very similar (55.4±6.1 µg/mL for 24h and 54.4±7.0 µg/mL for 48h). In addition, a remaining cell viability of approximately 20% was recorded after 24h and 48h of exposure to the

highest concentration tested (250 µg/mL) in PC content and MTS assays; however, RN uptake assayed showed higher cell viabilities (around 50% after 24h and 35% at 48h).

Results of the mutagenicity study

After exposure to CNa+, no significant changes were detected in any of the strains exposed to the clay mineral (Table I).

C30B showed significant increases in the revertant colonies compared to the negative control in TA98 with S9 at all concentrations assayed, while no change was recorded in the exposure without metabolic activation. However, no change was recorded in the other strains (Table I).

Similarly, Clay1 induced statistically significant enhancements in the number of revertant colonies in TA98 with S9 at all concentrations assayed. However, no change was observed for the same strain without S9 fraction or in the rest of the strains for this clay mineral (Table II).

In the exposure to Clay2, there was no change in the number of revertants and no indication of mutagenic activity in any of the treated strains up to the highest concentration (125 µg/mL), either in the presence or absence of S9 (Table II). The results showed that this clay was not potentially mutagenic in this bacterial assay.

In all exposures, positive controls produced statistically significant increases ($P<0.01$) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix. Moreover, no toxicity was observed at any concentration tested. This was measured as a decrease in background lawn and in revertant frequency.

Discussion

Clay minerals are naturally occurring inexpensive and eco-friendly materials and have found multifarious applications (Majeed et al., 2013). Despite the growing use of clays, very little research has been performed regarding their potential toxicity (Lordan et al., 2011). Mt, a widely used clay mineral, has been traditionally considered as safe to human (Liu et al., 2011); however, several authors have already pointed out toxic effects in different experimental models (Lordan et al., 2011; Sharma et al., 2010; Baek et al., 2012; Maisanaba et al., 2013,2014a,b; Houtman et al., 2014). Considering the large use of Mt in food packaging, due to the improvements developed in mechanical and barrier properties (Majeed et al., 2013), one of the most expected ways of exposure is the oral route. Once Mt passes the gastrointestinal epithelium and ends up in the blood circulation, particles are distributed around the body (Nemmar et al., 2002). Baek et al. (2012) demonstrated that Mt could be absorbed into the body within 2 h, but it did not significantly accumulate in any specific organ. Therefore, it can be easily found in blood vessels. Considering that endothelial cells could be the target of clay minerals, the present work studied for the first time the cytotoxic effects of different clay minerals in the human cell line HUVEC and their mutagenicity.

As far as we know, only Murphy et al. (1993) have studied the toxicity of clay minerals in HUVEC. They evidenced that aluminum silicate clay minerals (Mt, kaolinite and bentonite) are able to cause a dose-dependent release of fatty acids in human umbilical vein endothelial, neuroblastoma and oligodendroglial cells, being Mt the clay

mineral inducing the highest effect and HUVEC the cells exhibiting the highest release. In addition, only HUVE cells were susceptible to cell lysis, indicating a higher sensibility in comparison to the other cell lines (Murphy et al., 1993). Moreover, several authors have used other cell lines to study the toxic effects of CNa+ and C30B. In this sense, the absence of cytotoxic effects of CNa+ shown in the present work has been previously observed in U937 monocityc cells (Lordan and Higginbotham, 2012). Similarly, no cytotoxic effect was recorded in Caco-2 cells exposed up to 31.25 µg/mL of CNa+; however, significant decreases were observed when cells were exposed to the highest concentrations of this clay mineral (31.25-125 µg/mL) (Maisanaba et al., 2013,2014a). In addition, a concentration-dependent response in HepG2 cells exposed to CNa+, as well as with the modified clay Cloisite® 93A, was evident following 24 h of treatment with each of the clay minerals (Lordan et al., 2011). Also, Li et al. (2010) observed a slight decrease in CHO cells viability exposed to 62.5-1000 µg/mL nanosilicate platelets as measured with the MTT and lactate dehidrogenase (LDH) assays. In the case of C30B, Sharma et al. (2010) reported that this clay mineral induced about 40% cytotoxicity in Caco-2 cells at the highest concentration tested (226 µg/mL).

Considering that Clay1 and Clay2 are not commercially available, the results obtained can only be compared with previous studies performed in our laboratory. In the present work, no significant cytotoxic effects in HUVE cells exposed to Clay1 for 24 and 48 h was observed; however, all the endpoints assayed were significantly changed in exposure to Clay2. Caco-2 and HepG2 cells treated with Clay1 underwent significant reductions in the cytotoxicity assays only at 8 µg/mL (Houtman et al., 2014), likewise to

the results obtained in the present study for HUVEC in the MTS assay. In addition, the concentration and time-dependent reduction in the viability of HUVEC exposed to Clay2, was similar to those observed in Caco-2 and HepG2 cells (Maisanaba et al., 2013, 2014a). When EC50 values are compared, HUVEC showed a similar sensitivity to Caco-2 cells to the toxic effects of the studied clay minerals, and higher than HepG2 cells.

The present study demonstrates for the first time that cytotoxity and genotoxicity induced by exposure to clay minerals may not be always related. Clay1 did not induce any cytotoxic effects in HUVE cells, although it exhibited potential mutagenicity in TA98 *S. thyphimurium* strain. In contrast, Clay2 produced cytotoxicity in endothelial cells but no mutagenicity was recorded. However, CNa+ was not cytotoxic neither mutagenic. And finally, C30B showed positive results in both assays. Similarly, Sharma et al. (2010) observed no mutagenic activity in TA98 and TA100 exposed to sodium Mt (CNa+) and C30B in concentrations up to 141 µg/mL; CNa+ showed no cytotoxic effect but C30B did. Similarly, Li et al. (2010) reported no mutagenic potential in TA98, TA100, TA1535, TA1537 and TA102 exposed to exfoliate silicate nanoclay, although this material showed low cytotoxicity on CHO cells below 1000 µg/mL after 12 and 24 h of exposure.

However, our results correlate well with previous genotoxic studies performed in our laboratory with these clay minerals. No genotoxicity measured by the Comet assay was observed in Caco-2 cells exposed to C30B (Maisanaba et al., 2014a). However, this clay

induced DNA damage in the hepatic cell line HepG2 (Maisanaba et al., 2013). In addition, Clay2 showed DNA damage, measure by comet assay in Caco-2 and HepG-2 cells, although no genotoxicity was observed for Clay1 (Houtman et al., 2014). Moreover, genotoxic effects of different clays (bentonite kaolinite, quartz and exfoliated silicate nanoclay) on several experimental models have been previously reported (Gao et al., 2000; Geh et al., 2006; Li et al., 2010; Meibian et al., 2011).

In the present study, CNa+ was the only clay not showing any toxic effect. This finding suggests that the toxicity of organo-modified Mt was caused by the organo-modifier. In this sense, surface modification of clays has been commonly used to achieve a greater compatibility of the clay mineral with the polymer, and therefore reach higher exfoliation degrees, and in consequence better properties (Hetzer and De Kee, 2008). However, it has been suggested that the modification introduced in the Mt changes substantially its behavior in the experimental model (Maisanaba et al., 2014a). Baker et al. (2007) reported that the changes in clay structure and surface property may alter their response to blood-clotting. Therefore, it seems that the organo-modifiers used are the responsible for the higher toxic effects observed in the endothelial cells. However, other modifiers, such as amine or carboxyl groups, have shown to be useful for the development of safer nanosilica particles, exhibiting lower cytotoxicity and DNA damage than the unmodified ones (Yoshida et al., 2012). In this regard, amorphous silica nanoparticles have been shown to induce multinuclei and cell dysfunction in the human hepatic cell line L-02 (Wang et al., 2013).

In conclusion, the present work evidenced cytotoxicity in HUVE cells and genototoxic damage induced by organo-modified Mt-based clay minerals, whereas the unmodified clay mineral resulted to be safer at the concentrations tested. These results will contribute to the development of safer organoclays intended to be used in the food packaging industry. However, further studies are needed to assure the safety of these clays on human health.

Funding: this work was supported by the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study.

Acknowledgements: the authors wish to thank the Cell Culture Service of CITIUS (University of Seville) for providing technical assistance.

Conflicts of interest statement: the authors declare that there are no conflicts of interest.

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Legends to figures

Figure 1. Protein content, PC (a); Neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HUVECs after 24h and 48h of exposure to 0-62.5 µg/mL Cloisite ® Na+. All values are expressed as mean ± SD.

Figure 2. Protein content, PC (a); Neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HUVECs after 24h and 48h of exposure to 0-250 µg/mL Cloisite ® 30B. All values are expressed as mean ± SD. * significantly different from control ($P<0.05$) and ** very significantly different from control ($P<0.01$).

Figure 3. Protein content, PC (a); Neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HUVECs after 24h and 48h of exposure to 0-8 µg/mL Clay1. All values are expressed as mean ± SD. * significantly different from control ($P<0.05$).

Figure 4. Protein content, PC (a); Neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HUVECs after 24h and 48h of exposure to 0-125 µg/mL Clay2. All values are expressed as mean ± SD. * significantly different from control ($P<0.05$) and ** very significantly different from control ($P<0.01$).

Table 1

	Concentration (µg/mL)	TA97A		TA98		TA100		TA102		TA104	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
$C^{®}Na^{+}$	Negative controls	283±52	321±84	25±9	31±14	112±26	124±22	325±84	387±81	359±52	454±34
	7.8	177±63	177±63	27±8	45±18	121±27	105±27	349±81	390±73	214±52	350±43
	15.63	319±40	278±91	28±8	46±15	147±43	129±7	322±49	316±41	255±60	378±62
	31.25	278±63	207±44	24±17	30±8	91±6	158±16	297±63	320±71	248±24	266±35
	62.5	334±87	209±43	20±7	38±20	143±10	161±14	351±91	432±25	290±62	284±45
	125	244±55	255±30	19±3	23±3	149±40	149±33	282±90	305±35	314±75	314±75
	Positive controls	676±78**	676±10**	>1000**	>1000**	458±37**	395±4**	741±24**	774±11**	734±29**	741±46**
	DMSO	297±37	320±34	27±7	39±2	119±16	115±3	267±13	381±32	373±25	360±13
$C^{®}30B$	Negative controls	224±48	267±20	35±10	37±11	77±5	75±10	253±44	257±34	295±74	388±74
	15.63	258±59	323±49	35±19	872±56**	115±38	104±26	292±71	269±63	309±21	451±56
	31.25	118±20	225±34	36±17	930±99**	124±6	104±31	279±59	298±78	380±38	473±49
	62.5	303±68	229±62	20±4	930±99**	108±12	107±9	160±8	353±78	461±76	535±65
	125	245±48	275±55	26±6	>1000**	101±8	98±3	332±95	347±18	328±55	252±36
	250	289±33	279±47	36±22	766±48**	116±36	98±9	317±36	291±45	355±52	350±25
	Positive controls	649±14**	668±15**	>1000**	>1000**	372±7**	560±39**	486±58**	520±30**	594±49**	710±25**
	DMSO	236±8	239±33	29±8	39±2	96±5	101±13	248±4	249±19	265±35	352±10

Table I. Results of Ames test conducted with Cloisite® Na+ and Cloisite® 30B for three independent experiments. MilliQ water was used as negative control and DMSO as solvent for positive controls. Data are given as mean±SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/ TA98/ TA102/ TA104 without S9 mix: 2-NF (0.1 µg/plate) and TA100 without S9 mix: NaN_3 (1µg/plate). Positive controls for all strains with S9: 2-AF (20µg/ plate). ** very significantly different from control (P<0.01).

Table 2

	Concentration (µg/mL)	TA97A		TA98		TA100		TA102		TA104	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Clay1	Negative controls	216±28	206±12	21±3	38±14	85±16	80±18	183±25	216±45	252±45	285±45
	0.5	241±75	213±30	25±7	68±12*	91±26	87±17	142±45	166±12	221±51	228±54
	1	236±69	254±55	29±10	144±22**	79±15	79±24	180±25	230±43	252±69	236±54
	2	211±54	254±51	35±10	303±100**	70±11	88±18	189±48	173±66	270±73	367±69
	4	262±70	260±87	28±8	521±26**	89±21	85±18	281±72	242±67	309±51	341±86
	8	211±26	260±51	34±20	150±61**	80±15	89±32	218±84	202±41	295±47	323±83
	Positive controls	387±60**	485±86**	>1000**	>1000**	347±50**	411±120**	483±51**	541±29**	749±120**	>1000**
	DMSO	230±21	270±27	33±2	32±6	97±5	102±11	253±20	252±13	331±29	356±40
Clay2	Negative controls	241±15	255±30	33±12	29±10	73±8	79±11	252±28	217±30	292±68	252±47
	7.8	234±28	206±9	21±5	34±17	74±4	68±6	236±42	262±34	269±75	306±50
	15.65	255±56	278±50	34±7	35±1	78±9	67±12	247±81	236±73	306±95	282±94
	31.25	289±47	307±83	23±8	40±14	74±17	95±8	233±24	192±12	259±32	152±8
	62.5	278±63	228±28	24±4	35±18	59±4	67±1	207±36	181±23	265±55	228±76
	125	231±27	248±17	37±7	41±20	70±5	69±1	243±33	203±13	250±50	232±20
	Positive controls	642±14**	665±9**	>1000**	>1000**	549±86**	550±46**	494±67**	506±34**	615±18**	671±71**
	DMSO	223±55	287±28	35±3	36±4	98±2	104±14	238±10	259±19	333±11	349±10

Table II. Results of Ames test conducted with Clay 1 and Clay 2 for three independent experiments. MilliQ water was used as negative control and DMSO as solvent for positive controls. Data are given as mean±SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/ TA98/ TA102/ TA104 without S9 mix: 2-NF (0.1 µg/plate) and TA100 without S9 mix: NaN_3 (1µg/plate). Positive controls for all strains with S9: 2-AF (20µg/ plate). * significantly different from control ($P<0.05$) and ** very significantly different from control ($P<0.01$).

Figure 1

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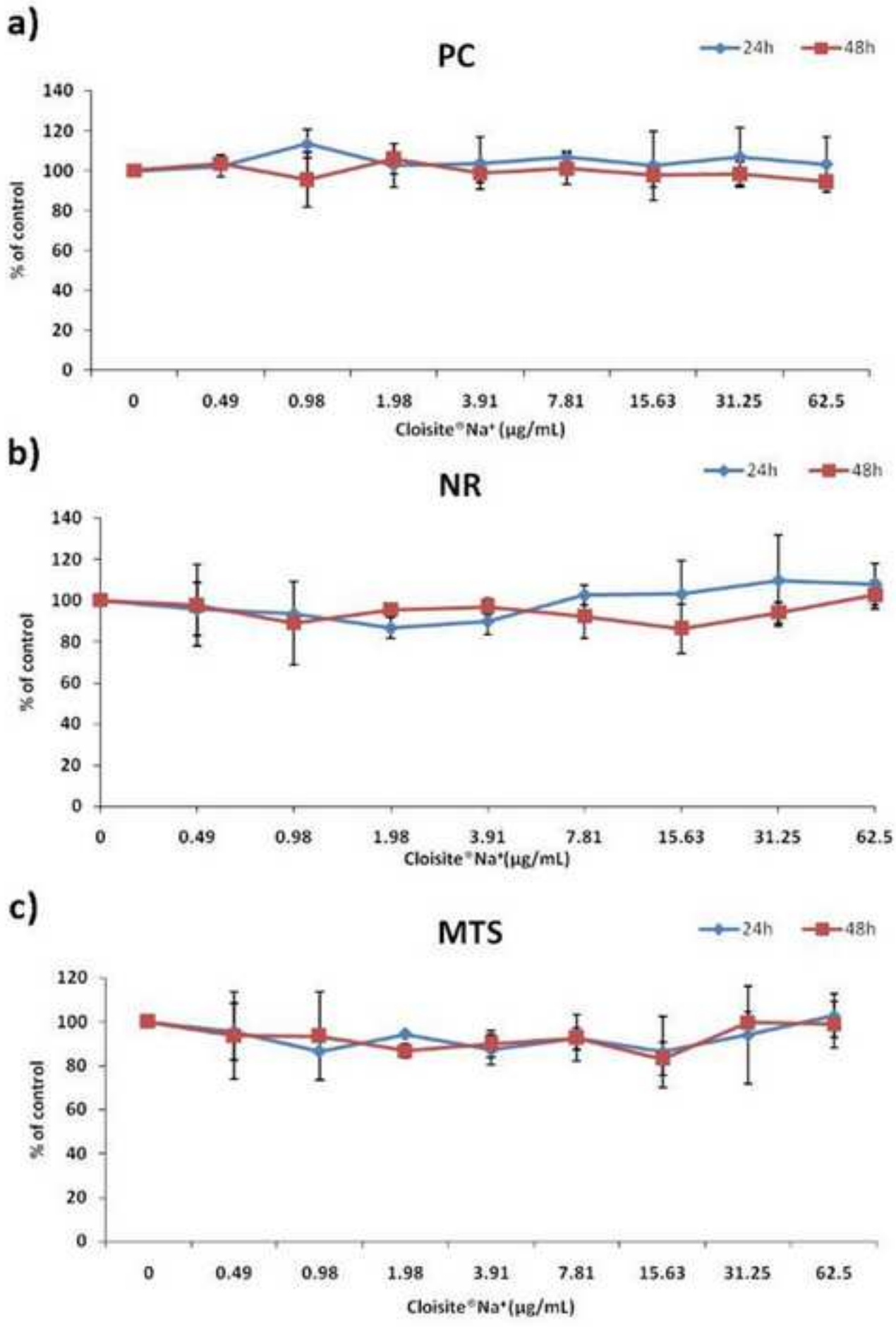

Figure 2

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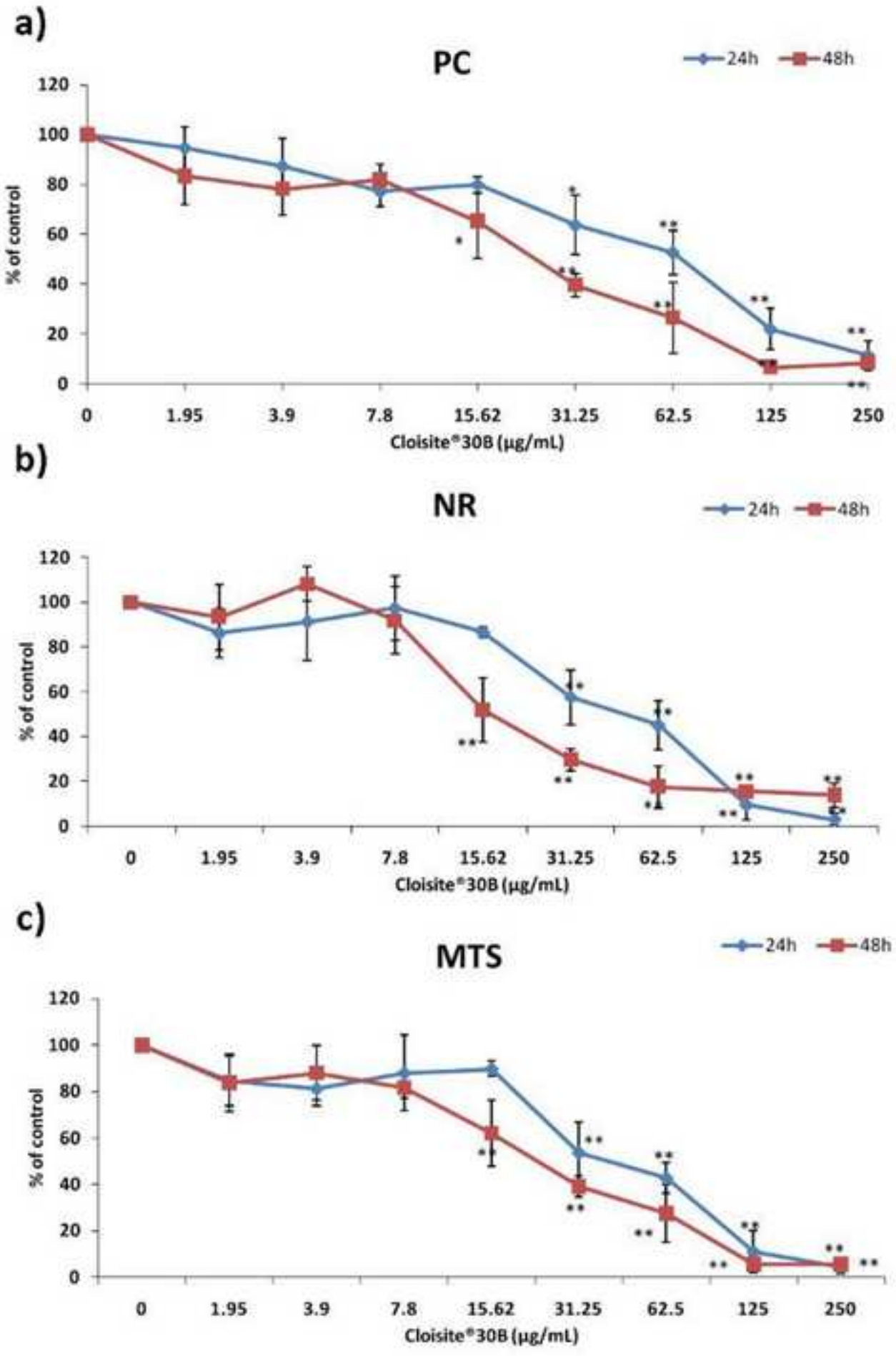

Figure 3

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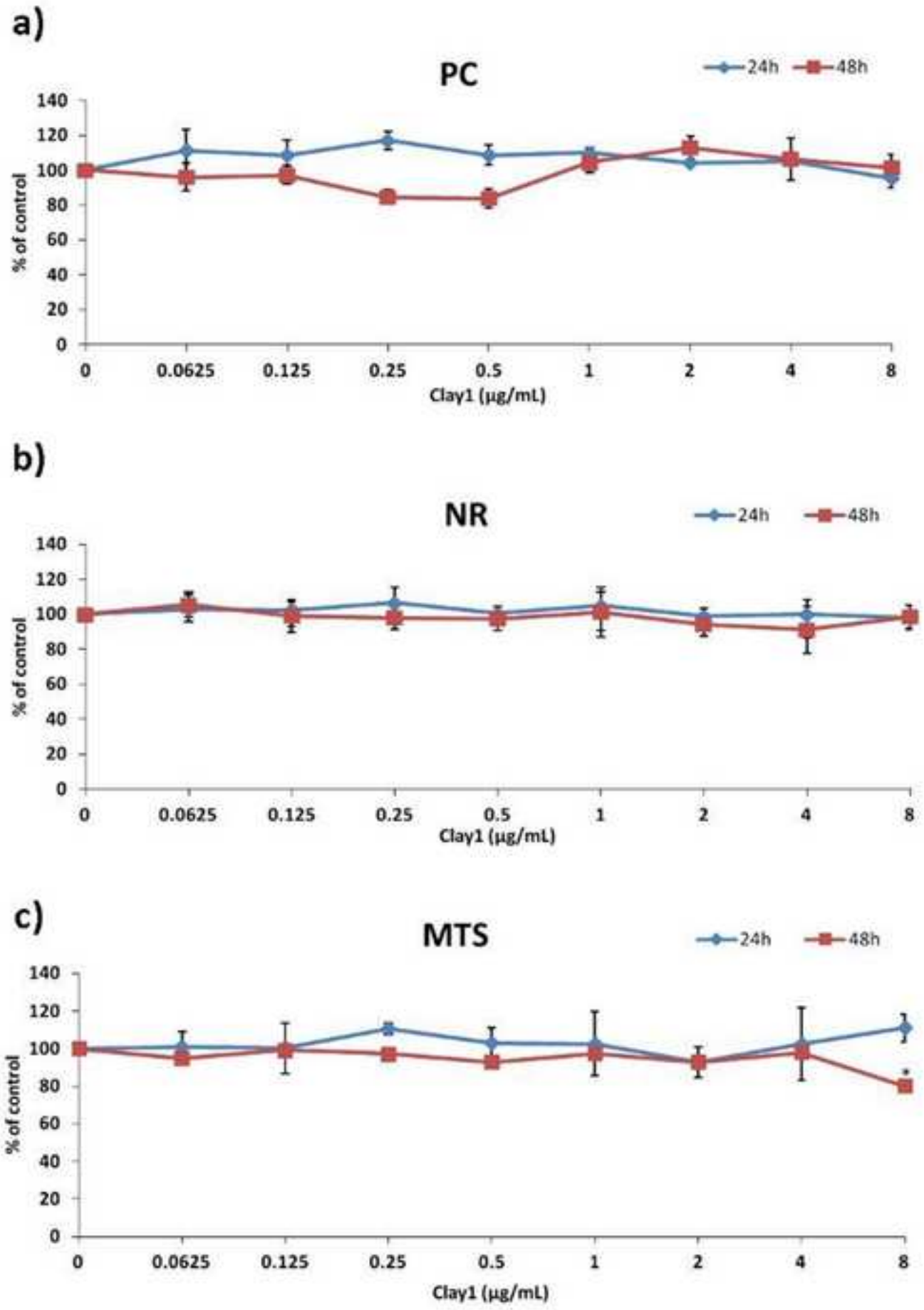

Figure 4

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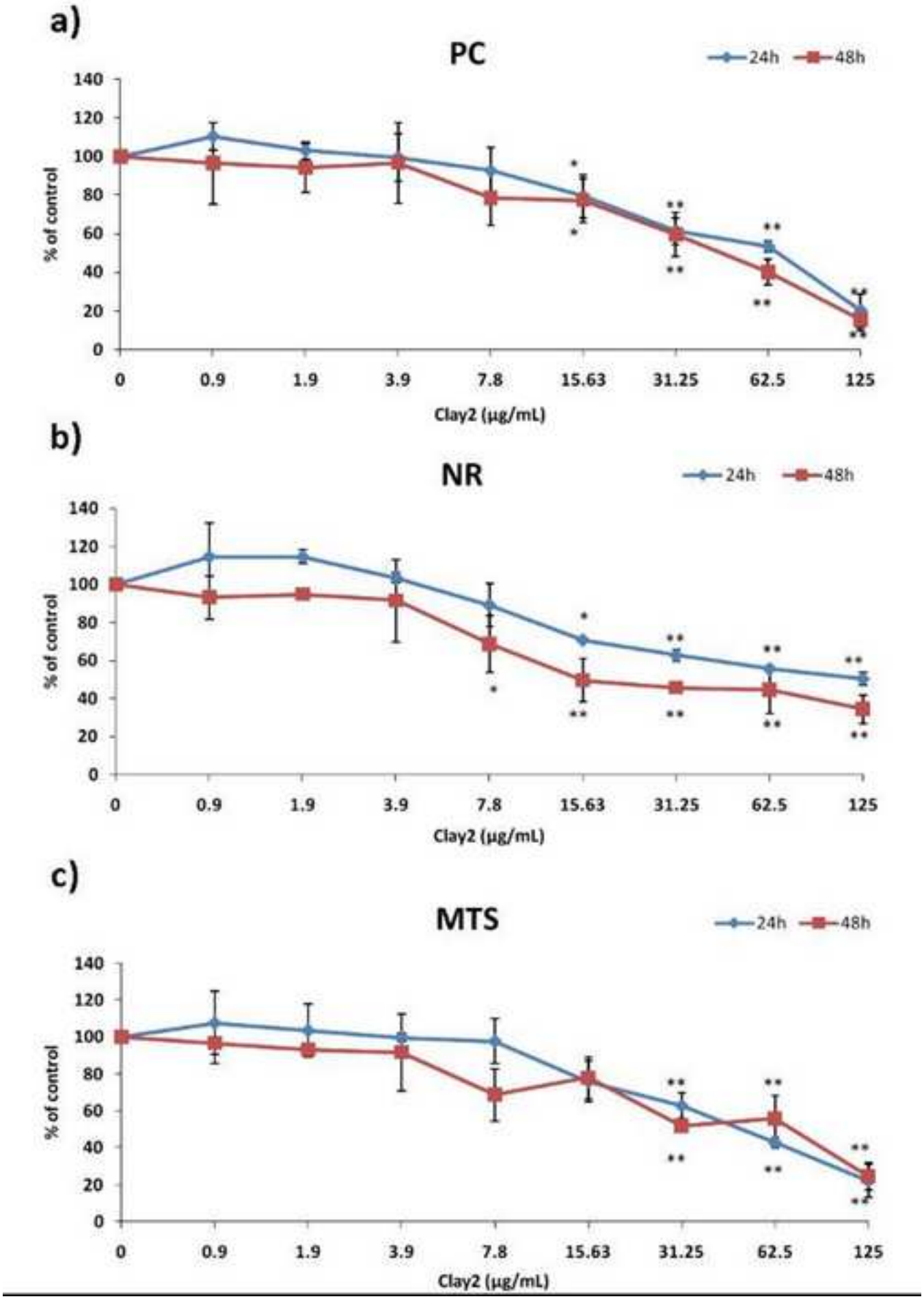

***Highlights (for review)**

Clay1 was not cytotoxic to HUVEC, although potential mutagenicity was found

Clay2 produced cytotoxicity in endothelial cells but no mutagenicity was recorded

CNa+ was not cytotoxic neither mutagenic

C30B showed positive results in cytotoxic and mutagenic studies

***Conflict of Interest**

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CAPÍTULO 9 / CHAPTER 9

Sara Maisanaba, Silvia Pichardo, María Jordá-Beneyto, Susana Aucejo, Ana M. Cameán, Ángeles Jos

CYTOTOXICITY AND MUTAGENICITY STUDIES ON MIGRATION EXTRACTS FROMA NANOCOMPOSITES WITH POTENTIAL USE IN FOOD PACKAGING

Food and Chemical Toxicology 66, 366-372, ***2014***

Elsevier Editorial System(tm) for Food and Chemical Toxicology
Manuscript Draft

Manuscript Number: FCT-D-13-02572R1

Title: Cytotoxicity and mutagenicty studies on Migration Extracts from Nanocomposites with potential use in Food Packaging

Article Type: Full Length Article

Keywords: migration; nanocomposites; clay; cytotoxicity; mutagenicity

Corresponding Author: Dr. Angeles Jos,

Corresponding Author's Institution: University of Sevilla

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Silvia Pichardo; María Jordá-Beneyto; Susana Aucejo; Ana M Cameán; Angeles Jos

Abstract: Clays are used in the food packaging industry to obtain nanocomposites. The use of these new materials is a concern, because they could reach consumers by oral exposure through possible migration, and potential toxic effects could be derived. In the present study, several in vitro basal cytotoxicity and mutagenicity tests on migration extracts obtained from a nanocomposite material with poly (lactic) acid (PLA) and two modified clays, Clay1 and Clay2, are shown. Migration extracts in distilled water showed values of 0.1±0.2 mg/dm2 in all samples. Also, the content of characteristic metals of the clays structure (Al, Ca, Mg, Fe, Si) was studied and no statistical differences were observed. For the cytotoxicity assays, the human intestinal Caco-2 and human liver HepG2 cells were selected. Cells were exposed to concentrations between 2.5%-100% extracts determining three different biomarkers of cellular viability. No significant differences were observed in the cytotoxicity assays. Finally, mutagenicity was evaluated by the Ames test and resulted in the absence of mutagenic response at all the concentrations assayed. Taking in account all above mentioned, these new materials show a good profile for their use in food packaging although further research is still needed.

Response to Reviewers: Response to reviewers' comments
Authors: Thank you very much for your thorough revision that will contribute to improve the quality of our manuscript.
Your comments and queries have been considered as follows:

Reviewers' comments:

Reviewer #1: The number of figures is excessive, must be reduced. A example by each kind of assay is enough.
Authors: following your suggestion we have deleted the graphs corresponding to HepG2 cells. The total number of figures has been reduced to 3.

2.7. Analysis of metal content in the migration extracts.
Indicate whether migration extracts were filtered before analysis and if insoluble solids were detected. In the case of insoluble solids, were metals in that form solubilized before analysis.

Authors: Thank you for your comment. Migration extracts were not filtered before the analysis because the technician of the Microanalysis Service did not observe any suspended material and considered that the samples were clean enough to perform the analysis directly. We have included in M&M section this point.

2.8 and 2.9 Citotoxicity assays and Ames test.
Why were not prepare and use standard suspensions of nanoclays to establish if they are cytotoxic or mutagenic?
Authors: Thank you for your comment. Clays before being imbued in the polymeric matrix have no nanometric size. We already tested the cytotoxicity of Clay1 and Clay2 before this process and found that Clay1 was not cytotoxic at the concentrations assayed and Clay2, on the contrary was. In our opinion, transforming artificially both clays into nanoclays would have introduced differences in comparison to the real production procedure (solvents, etc.) that could have an influence in the final (toxic) response. Therefore, we used this procedure because is the nearest to the real exposure scenario.

3.0. Results
Was the residue analyzed to see if nanoparticles were in it?
Authors: No, taking into account that the results of the migrations assays were 0.1 ± 0.2 mg/dm2, much lower than the limits established by law we did not do the analysis. Moreover, even the European Food Safety Authority recognizes that currently it is not possible to routinely determine engineered nanomaterials in situ in the food (EFSA 2011, EFSA Journal. 9(5): 2140).

Reviewer #2: In this manuscript, the author evaluated "cytotoxicity and mutagenicty on migration extracts from nanocomposites with potential use in food packaging". The study has a high significance and medium novelty, The study design is very good. However, based on the results, the conclusion "the nanocomposites assayed seem to be safe for their use in food packaging applications" can not be made. This conclusion should be narrowed.
Authors: Thank you for your comment. We have rewritten the conclusion taking into account your suggestion.

Angeles Jos
Area of Toxicology. Faculty of Pharmacy. University of Seville
C/Profesor García González 2, 41012 Seville, Spain
Tel.: +34-954556762; fax: 34-954556422.
E-mail address: angelesjos@us.es

12th January, 2014

Dear Editor,

Thank you very much for giving us the opportunity to submit a revised version of our manuscript entitled **"Cytotoxicity and mutagenicty studies on Migration Extracts from Nanocomposites with potential use in Food Packaging "** for its publication in Food and Chemical Toxicology.

We have answered the reviewers' comments and we hope the new version satisfies them.
I am looking forward to receiving a positive answer from you.

Sincerely,

Angeles Jos

Conflict of Interest Statement

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Cytotoxicity and mutagenicty studies on Migration Extracts from Nanocomposites with potential use in Food Packaging

Sara Maisanaba[1], Silvia Pichardo[1], María Jordá- Beneyto[2], Susana Aucejo[2], Ana M. Cameán[1], Ángeles Jos[1]*

[1]Area of Toxicology, Faculty of Pharmacy, Universidad de Sevilla, Profesor García González n° 2, 41012 Seville. Spain.

[2]Packaging, Transport, & Logistics Research Institute (ITENE), C/ Albert Einstein, 1. Parque Tecnológico. Paterna, Spain

*Corresponding author:

Angeles Jos

Area of Toxicology, Faculty of Pharmacy, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: angelesjos@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Highlights:

- Novel nanocomposites of PLA and modified clays were performed for food packaging
- Values obtained in the migration tests of the nanocomposites were below legislation limits
- Cytotoxic effects were not observed in cells lines exposed to the migration extracts
- Nanocomposites extracts did not show mutagenic activity
- The migration of metals was lower than the limits established by the EU legislation

Abstract

Clays are used in the food packaging industry to obtain nanocomposites. The use of these new materials is a concern, because they could reach consumers by oral exposure through possible migration, and potential toxic effects could be derived. In the present study, several *in vitro* basal cytotoxicity and mutagenicity tests on migration extracts obtained from a nanocomposite material with poly (lactic) acid (PLA) and two modified clays, Clay1 and Clay2, are shown. Migration extracts in distilled water showed values of 0.1±0.2 mg/dm^2 in all samples. Also, the content of characteristic metals of the clays structure (Al, Ca, Mg, Fe, Si) was studied and no statistical differences were observed. For the cytotoxicity assays, the human intestinal Caco-2 and human liver HepG2 cells were selected. Cells were exposed to concentrations between 2.5%-100% extracts determining three different biomarkers of cellular viability. No significant differences were observed in the cytotoxicity assays. Finally, mutagenicity was evaluated by the Ames test and resulted in the absence of mutagenic response at all the concentrations assayed. Taking in account all above mentioned, these new materials show a good profile for their use in food packaging although further research is still needed.

Keywords: migration, nanocomposites, clay, cytotoxicity, mutagenicity

1. Introduction

Nanotechnology is a science that involves research and technology development at the atomic, molecular and macromolecular levels, aimed at creating and using structures, devices, and systems with novel properties and functions based on their small size (Farhang, 2009). Nanotechnology has opened up new avenues of research and development in numerous fields, including medicine, cosmetic and agro-food area (Chaundry et al., 2008). The application of this technology in food and agriculture plays an important role in improving production, processing, storage, transportation, traceability, safety and security of food (Chaundry et al., 2008). Among the wide range of applications of nanomaterials in the food industry, the one with the main development in the near future is their employ as food contact materials (AESAN, 2010). Nanomaterials have demonstrated to possess novel characteristics that can be applied in developing new packaging with better properties than packaging produced with micromaterials (Pereira de Abreu et al., 2007). The resulting materials, which are frequently polymers with nanoparticles incorporated in their structure, present improvements of barrier properties, such as thermal, mechanical or permeability characteristics, providing innovative solutions to increase the performance of the polymers (Silvestre et al., 2011). Therefore, nanotechnology plays an important role in the preservation of perishable food.

Among the most commonly used materials in food packaging are nanocomposites based on organic polymers and inorganic clay minerals (Pereira de Abreu et al., 2007). These clays consist of silicate nanoplates whose structure presents platelet morphology. Once the clays have been incorporated into the polymer, the resulting platelets force gases and other

external agents to follow a tortuous path through the material, decreasing and slowing the contact with the product obtaining more resistant plastics (Pereira de Abreu et al., 2007; AESAN, 2010).

The Technological Institute of Packaging, Transport and Logistic (ITENE) is developing modified clays based in montmorillonite (natural phyllosilicate), one of the main mineral clays used to be incorporated in food packaging. The use of functionalized clays, immersed and dispersed in the polymers for packaging results in nanocomposite materials, conformed by platelets with submicrometer dimensions, except for their thickness, which present nanometric dimensions (Hatzigrigoriou et al., 2011). These dispositions allow preventing the exchange of oxygen, carbon dioxide and humidity with the food, maintaining its organoleptic and nutritional characteristics for a longest period of time (Farhang, 2009).

The nanoscale formulation of materials change physicochemical properties compared with the bulk or microstructurated material. Smaller size, higher surface to volume ratio and greater reactivity are properties that confer advantages to the use of nanomaterials. Moreover, the toxicity and toxic kinetic profile also change, since it cannot be inferred from data of their non-nanostructured homologous. Therefore, the potential risk for the health and the environment could be modified (EFSA, 2009), making necessary to investigate the toxicity profile of these modified clays, even more, taking into account the scarce toxicological information available so far.

Considering that the consumer exposure to these nanoclays is likely since they are present in the food packaging, it is interesting to evaluate not only the microstructured clay, but also

the imbued nanomaterial in the polymer, due to the possible migration to the food product (Guillard et al., 2010; EFSA, 2011; Song et al., 2011).

The EU Commission Regulation N° 10/2011 and the Council Directive 82/711/EEC collect the aspects related to plastic materials and articles intended to come in contact with food and foodstuffs, as well as the simulants used in the migration assays, and it states that the risk assessment should cover the potential migration under worst foreseeable conditions of use and the toxicity. Moreover, the Commission Regulation EU nº 10/2011 also establish an overall migration limit of 10 mg/dm^2, this is plastic materials and articles shall not transfer their constituents to food simulants in quantities exceeding 10 milligrams of total constituents released per dm^2 of food contact surface.

As nanoclays are substances that could be potentially present in food, the oral pathway would be the most commonly entry for these substances in the organism. Thus, it would be interesting to establish the toxicological profile of the migration extracts with *in vitro* assays, and thereby contribute to the risk assessment of nanomaterials used in food industry, claimed by the European Food Safety Authority (EFSA, 2011). In this sense, the present study provides a toxicological evaluation of two migration extracts obtained from nanocomposite materials with PLA and two modified clays developed by ITENE, Clay1 and Clay2, through several cytotoxicity assays on two specific and target cell lines (Caco-2 and HepG2 cells, from intestinal and liver origin, respectively) and a mutagenicity assay, the Ames test.

Moreover, the presence of characteristic metallic elements of the clays structure (Al, Ca, Fe, Mg, Si) was also determined in the migration extracts.

2. Materials and Methods

2.1. Supplies and Chemicals

Minimal Essential Medium (MEM) powder (1x) with Earle´s Salts was obtained from PPA laboratories GmbH (Austria) and other cell culture reagents were supplied from Gibco (New Zealand). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Cell Culture

Caco-2 cell line derived from human colon carcinoma (ATCC Nº HTB-37) was maintained at 37ºC in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire, Spain), in a medium consisting of Minimum Essential Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 mg/mL gentamicin, 1.25 mg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. The cells were used at passages between 12-21. HepG2 derived from a liver hepatocellular carcinoma (ATCC Nº HB-8065) was maintained at similar conditions in EMEM supplemented with 10% FBS, 100 U/mL penicillin and 100µg/mL streptomycin. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested each two-three days with 0.25% trypsin. The cells passes used were 16-32.

2.3. Clays and polymer

Purified sodium montmorillonite (MMT) (Cloisite® Na^+), with cationic exchange capacity (CEC) of 116meq/100g was purchased in Southern Clay Products (COMITEX, S.A. Spain).

Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) ($C_{19}H_{42}BrN$, 364.46 g/mol, 98%) was supplied from CymitQuímica S.L. (Spain), acetylcholine chloride (ACO) ($C_7H_{16}ClNO_2$, 181.66 g/mol, ≥99%) was provided by Sigma-Aldrich (Spain). Polylactide (PLA) pellets were purchased from Cargill Dow (NatureWorks ® PLA 7032 polymer) for the preparation of filled biopolymers (Fig.1).

2.4. Organo-modified nanoclays

Organo- modified clays have been developed and characterized by ITENE based on previous works (Jordá Beneyto et al., 2008; Jordá et al., 2009). Briefly, two different organo-modified clays were prepared by a cation-exchange method, which consists on a displacement of the sodium cations of Cloisite® Na^+ with the ammonium cations of the above mentioned salts, obtaining Clay1 and Clay2. Both clays were characterized by Fourier Transform InfraRed (FTIR), Wide-angle X-ray Diffraction and Termogravimetric Analysis (TG) following the methods described in Jordá- Beneyto et al. (2013).

2.5. Nanocomposites preparation

Different PLA nanocomposites samples have been obtained with the prepared modified clays, Clay1 (MMT + HDTA) and Clay 2 (MMT + HDTA + ACO) with 4 % clay content. For this purpose a twin screw extruder DSE 20/40 (COPERION) was used. The twin screw extruder was used to prepare the samples through melt- direct intercalation. PLA pellets (dried overnight at 90ºC and vacuum for 2 hours (MANN HUMMEL PicolinoDehumidifier)) were blended with 4% in weight of Clay1 and Clay2, respectively. Bottles were obtained by injection stretch blow moulding (ISBM). An important part of the process is the mechanical

stretching of the preform during the moulding process, which helps to increase the impact resistance of the container and also helps to produce a very thin walled container, affecting final mechanical, thermal and chemical properties of the nanocomposite material. The thermal, mechanical and barrier properties of the materials obtained were analyzed following the techniques described in Jordá-Beneyto et al. (2013).

2.6. Migration Extracts

Migrations extracts of both nanocomposites were provided by ITENE. Migration tests were carried out according to UNE-EN 1186-9:2002 (AENOR, 2002). The samples, bottles of 150 mL of nanocomposite materials PLA+ Clay 1 and PLA+ Clay 2, (123,655 dm^2 and 590-598 µm thick) were filled with the simulant chosen according to the law (EU Commission Regulation N° 10/2011 and Council Directive 82/711/EEC), distilled water, at the specific conditions established by the regulations (Temperature(C°): 40; Time (days): 10). Then, all volume of simulants were poured into cell glasses and exposed to heat in order to the simulants were evaporated. Afterwards, the residues were weighed and compared with control cells glasses (without simulants) and the presented difference is the global migration from the simulants. Moreover, nanocomposites of PLA -Clay1, and, PLA -Clay2, were also filled with the specific culture medium of each cell line used as simulant. Each sample was evaluated by triplicate.

2.7. Analysis of Metal Content in the Migration Extracts

The presence of characteristic metals of the clays structure in PLA-Clay1, PLA-Clay-2 and only PLA (as control) unfiltered extracts was analyzed. Al, Fe and Mg were quantified by (ICP-MS) (Agilent 7500C, Agilent ICP-MS Systems, USA). Also, Si and Ca were studied by (ICP-OES)

(Horiba Jobin Yvon 2). These determinations were carried out in the Microanalysis service of the Research, Technology and Innovation Center of University of Seville (CITIUS) based on standard operating protocols.

2.8. Cytotoxicity assays

Caco-2 and HepG2 cells were seeded in a 96-well tissue-culture plates at 7·x 10^5 cells/mL, and incubated at 37°C for 24 h prior to exposure. Cells were exposed for 24 and 48h to the following concentrations: 2.5, 5, 10, 20, 40, 60, 80 and 100% of the migration extracts of PLA-Clay1 and PLA-Clay2 reconstituted with powder culture medium. Exposures were also performed with culture medium used as simulant in the migration tests. The basal cytotoxicity biomarkers assayed were: tetrazolium salt reduction (MTS), neutral red cellular uptake (NR) and protein content (PC).

MTS reduction is carried out by dehydrogenases enzymes present in mitochondria. MTS reduction was measured in intact cells according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt) tetrazolium compound added to the medium is bioreduced by cells into a colored formazan product soluble in culture medium which is directly measured with the spectrophotometer at 490 nm after 3 h of incubation in the dark. The lysosomal uptake neutral red (NR) was performed according to Borenfreund and Puerner (1984). The absorbance of the extracted NR from the viable cells was measured in a spectrophotometer at 540 nm. Protein content (PC) was quantified in situ, according to the procedure given by Bradford (1976). The culture medium was replaced by 200 mL NaOH and after 2 h incubation

at 37 ºC, 180 µL was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature absorbance was read at 620 nm.

2.9. Ames test

The incorporation version of the Ames test was performed according to the recommendations of Maron and Ames (1983) and following the principles of OCDE guideline 471 (1997). Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used for the assay. Cultures of each tester strain were prepared from their main strain plates, and use in late exponential growth phase. Each test substance was assessed in three independent experiments, each conducted in absence and presence of S9 metabolic activation system from rats livers (Aroclor 1254, Sigma, Spain), using triplicate plates for each test substance concentration. Both extracts were tested in five different concentrations (20%, 40%, 60%, 80%, 100%). Briefly, 0.1mL of test substance, 0.1mL of bacterial culture and 0.5mL of S9 mix, were added to 2mL molten agar at 42° and poured onto Vogel Bonner-E minimal glucose agar plates. Plates were inverted and incubated 65-70 h at 37°C in the dark. Revertant colonies were counted and background lawn was inspected for signs of toxicity or compound precipitation. 2-Nitrofluorene (2-NF) (0.1 µg/plate) and sodium azide (NaN_3) (1 µg/plate) were selected as positive controls.

2.10. Calculations and statistical analysis

All experiments were performed three times and at least in triplicate per concentration. Data of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control group, being unexposed cells. Statistical analysis was

carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests using GraphPadInStat software (GraphPadSoftware Inc., La Jolla, USA). The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlett´s test, respectively. Differences were considered significant from $p < 0.05$.

3. Results

3.1. Migration Extracts of PLA-Clay1 and PLA-Clay2.

Overall migration tests were carried out in 150 mL bottles of nanocomposite materials (123.655104 cm^2 total area) according to UNE-EN 1186-9 standard (2002). The results showed values of 0.1 ± 0.2 mg/dm^2 for each sample.

3.2. Metal Content on the Migration Extracts

Aluminum contents of the control and PLA-Clay2 extract were lower than the limit of detection (LOD) and quantitation (LOQ) of the technique, respectively (Table 1). However PLA-Clay1 extract showed 0.7± 0.2 µg/L Al. Ca contents could not be quantified in any sample. Similarly, Fe contents were ≤ LOD in all the samples. Mg contents in PLA-Clay1 showed the highest value in comparison to PLA and PLA-Clay2 extracts. Finally, Si was the most abundant element, showing similar contents in PLA and PLA-Clay1, but lower content was presented in PLA-Clay2. Overall, the metal levels found were very low and always under the currently values permitted by the European Union (Commission Regulation EU nº 10/2011).

3.3. Basal Cytotoxicity assays

3.3.1. Migration Extract of PLA-Clay1 in Caco-2 and HepG2 cell lines.

The migration extract of PLA-Clay1 showed no cytotoxic effects on Caco-2 cells in the three biomarkers assayed. In the MTS assay, no significant differences with respect the control were observed in any concentration assayed after 24 and 48 h exposure (Fig. 2A). Similar results were obtained with neutral red uptake (Fig. 2B); no significant differences were observed from 0% to 100% after 24 and 48 h of exposure. Regarding protein content, the same pattern was followed in this endpoint; however, lower results were observed after 48h of exposure respect to 24h (Fig.2C).

The results obtained in the MTS, NR and PC assays for the HepG2 cell line exposed to the migration extract of PLA-Clay1 did not show significant differences with respect to control group in any of the concentrations assayed after 24 and 48 h of exposure (data not shown~~Fig. 3~~).

3.3.2. Migration extract of PLA-Clay2 in Caco-2 and HepG2 cell lines.

When Caco-2 cells were exposed to the migration extract of PLA-Clay~~-~~2 similar results were observed to those obtained with the migration extract of PLA-Clay1 in the same cell line. No evidence of cytotoxicity was observed in any of the biomarkers assayed (Fig. 34).

The results observed when HepG2 cells were exposed to the migration extracts of PLA-Clay~~-~~2 did not show significant differences at the range of concentrations assayed after 24 and 48 h of exposure in the MTS metabolization, NR uptake and PC assay~~-~~(data not shown) ~~(Fig. 5)~~. ~~However, in the three biomarkers, a higher effect was observed at 48h of exposure, although it was not significantly different from the control group.~~

The absence of cytotoxic effects was also observed when cells were exposed to the cell culture medium used as simulant.

3.4. Ames test

After exposure to PLA-Clay1 and PLA-Clay2 there was no increase in the number of revertants and no indication of mutagenic activity in any of the treated strains up to the highest concentration (100%) of the extracts, either in the presence or absence of S9 (Table 2). Toxicity, measured as a decrease in background lawn and in revertant frequency, was not observed at any concentration tested. Positive controls produced statistically significant increases (p<0.01) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix. It was concluded that both extracts were not genotoxic in this bacterial assay.

4. Discussion

There is a major concern about food safety of packaging materials relative to the possible migration of packaging constituents, even more when the packaging is produced by a novel technology. In the present research work, in order to evaluate novel nanocomposite materials intended to be used in food packaging, several *in vitro* toxicity studies have been performed with their migration extracts.

The results obtained in the migration tests with the two nanocomposite materials employed are the first reported for these compounds since they have been developed by our research group and they are not yet commercialized. The values obtained are below the migration

limits required by the legislation (Commission Regulation (UE) Nº 10/2011), which establishes that the plastic materials should not release more than 10 mg of substance per 1 dm^2 of the plastic surface. Other researchers have evaluated several modified polymers with other different modified clays, including in some cases MMT. Thus, Schmidt et al. (2012), Busolo et al. (2012) or Avella et al. (2005) also found migration levels in accordance with the EU legislation. Nevertheless, Commission Regulation (UE) Nº 10/2011 establishes that these substances (nanoparticles) should be assessed on a case-by-case basis as regards their risk until more information is known about such new technology.

Cytotoxicity assays on Caco-2 and HepG2 cells exposed to migration extracts of the nanocomposite materials showed similar results in both cases. No significant differences were observed in the range of concentrations assayed, and total cell viability was unaltered in all cases. These results confirm that the migration extracts of the nanocomposite materials containing Clay1 and Clay2 are not cytotoxic at the conditions assayed. This could be considered an important start-point for the potential commercial launch of these nanocomposites for food packaging applications considering that consumer's safety is requested.

Scarce studies have reported so far regarding to cytotoxic effects induced by extracts of nanocomposite materials and modified montmorillonite clays. Rodrigues da Silva et al. (2010) observed the biocompatibility and cytotoxicity of the biodegradation products of two modified polyurethanes (PUD) intended to medical applications; PUD5 that contains poly (caprolactone) as soft segment, and PUD6, which has poly (caprolactone) and poly (ethylene glycol) as soft segment, with montomorillonite. After 48h, at 3:10 ratio, the cell viability of

the human retinal pigment epithelial cell (ARPE-19), measured by mitochondrial conversion of the tetrazolium salt, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), decreased in the modified polyurethanes and nanocomposites supplemented mediums (94,2 ± 3,26 %), although the statistical analysis showed no significant differences on the viability of ARPE-19 cells cultures in the PUD supplemented and control mediums. Fukushima et al. (2012) also studied the cytotoxicity of the degradation products of poly(butylenesadipate-co-terephthalate)(PBAT) modified with modified and unmodified montmorillonites, and other two clays, modified and unmodified fluorohectorites and unmodified sepiolite (intended to medical and industrial applications). This research group evaluated the biocompatibility of PBTA and nanocomposites with 10% of clay content trough an *in vitro* cytotoxicity and a protein adsorption test, chosen as experiment model fibroblast 929 cells. Cell viability and morphology were measured using MTT assay and inverted microscopy, respectively, after 0, 24, 72 and 120 h of incubation with a dilution of each extract. MTT results revealed no visible reduction in viability between the negative control and experimental groups at all times assayed. At the same time, PBAT and nanocomposites showed similar increases in the relative cell growth depending on the time of incubation, evaluated by direct observation from micrographs, evidencing that the addition of the nanoparticles did not induced further cytotoxic effects in L929 cells. In addition, the cytotoxicity of degradation products of chitin based polyurethane bio-nanocomposites (PUBNC), intended to food packaging, and prepared with bentonite nanoclay enriched in montmorillonite (MMT) and other compounds have been investigated by Zia et al. (2011). The cytotoxicity was measured by live/dead staining of L-929 fibroblast cells exposed to different concentrations of degradation products of the nanocomposite material, 0, 1, 2, 4

and 8% clay content showing significant differences in the groups containing 2-8% of bentonite nanoclay with respect to control and 1% bentonite content group.

Interestingly, the cytotoxicity results obtained with the migration extracts from nanocomposites containing Clay1 and Clay2 differs from the *in vitro* evaluation of the initial microstructured clays. In previous studies carried out in our laboratory, no cytotoxic effects were observed in HepG2 cells exposed to Clay1 from 0 to 8 µg/mL (no higher concentrations could be tested due to its hydrophobicity) after 24 and 48 h (Houtman et al., 2013). However, our research group observed significant cytotoxicity effects on HepG2 exposed to Clay–2, in a range of 0 to 125 µg/mL during 24 and 48h, obtaining mean effective concentration (EC_{50}) values for the MTS assay of 88 and 51 µg/mL, respectively (Houtman et al.,2013, Jordá-Beneyto et al., 2013). Similarly, no effect was recorded in Caco-2 cells exposed to Clay1, although in the case of Clay2, EC50 values of 34 and 18.5 µg/mL after 24 and 48h, respectively were found for the PC. Overall, Clay1 did not show cytotoxic effects in any case, whereas Clay2 in its microstructured formulation seems to be toxic on HepG2 and Caco-2 cells; but no toxicity was found once the clay was imbued in the polymer.

A test for induction of gene mutations in bacteria is among the core set of toxicological tests required in the Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorization (European Commission 2001). In the present study any of the extracts have shown mutagenic potential with and without metabolic activation. Sharma et al. (2010) also observed no mutagenic activity in TA98 and TA100 exposed to filtered and unfiltered

suspensions of purified sodium MMT (Cloisite® Na^+) and the commercial organoclay Cloisite® 30B. Also, Li et al. (2010) evaluated the mutagenic potential of exfoliated silicate nanoclay in TA98, TA100, TA1535, TA1537 and TA102. In this case, revertants colonies were not present in any strain assayed compared to the negative control plates, in presence or absence of S9. The absence of a toxic response in this test is in accordance with the results obtained in the cytotoxicity studies.

Regarding the study about the presence of metals in the migration extracts, no significant differences were observed in the analysis. However, Avella et al. (2005) studied the metals migration from nanocomposites films with potato starch and montmorillonite to lettuce and spinach. No differences were observed in Fe and Mg content in the vegetables, while a higher Si content was observed. This finding could be easily attributable to the fact that clay nanoparticles are mainly composed of this element. On the other hand, Busolo et al. (2012) investigated the migration of Al and Fe from polyolefin nanocomposite films containing an iron modified kaolinite with two simulants, water and isooctane, and, no differences were observed in any samples assayed.

5. Conclusion

The global migration obtained for the nanocomposites assayed was below the legislation recommendations, as well as their metallic content. Moreover, no cytotoxic and genotoxic effects were observed with the migrations extracts. ~~Thus, the nanocomposites assayed seem to be safe for their use in food packaging applications. However,~~ Ff~~f~~urther toxicological

studies are needed to confirm the safety of these nanocomposites for their use in food packaging applications~~confirm this hypothesis~~.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, the Cell Culture Service and Microanalysis Service of Centro de Investigación and Tecnología e Innovación de la Universidad de Sevilla (CITIUS), Consuelo Fernández Rivas and Jose María Bermúdez for providing technical assistance.

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Table captions

Table 1. Al, Ca, Fe, Mg and Si content in the migration extracts from PLA, PLA-Clay1 and PLA-Clay2 bottles (all the concentrations are expressed in µg/L).

Table 2. Results of Ames test conducted with PLA-Clay1 and PLA-Clay2 extracts for three independent experiments. Water was used as negative control and DMSO as solvent for positive controls. Data are given as mean±SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/ TA98/ TA102/ TA104 with and without S9: 2-NF (0.1 µg/plate) and TA100 with and without S9: NaN_3 (1µg/plate). ***p*<0.01 significantly different from control.

Figure captions

Figure 1. Structure of the quaternary ammonium salts: Acetylcholine chloride (a) and Hexadecyltrimethylammonium bromide (b) used to modify the montmorillonite.

Figure 2. ~~-~~Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in Caco-2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay1 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).

~~Figure 3. Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in HepG2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay1 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).~~

Figure 34. Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in Caco-2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay2 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).

~~Figure 5. Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in HepG2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay2 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).~~

1. Introduction

Nanotechnology is a science that involves research and technology development at the atomic, molecular and macromolecular levels, aimed at creating and using structures, devices, and systems with novel properties and functions based on their small size (Farhang, 2009). Nanotechnology has opened up new avenues of research and development in numerous fields, including medicine, cosmetic and agro-food area (Chaundry et al., 2008). The application of this technology in food and agriculture plays an important role in improving production, processing, storage, transportation, traceability, safety and security of food (Chaundry et al., 2008). Among the wide range of applications of nanomaterials in the food industry, the one with the main development in the near future is their employ as food contact materials (AESAN, 2010). Nanomaterials have demonstrated to possess novel characteristics that can be applied in developing new packaging with better properties than packaging produced with micromaterials (Pereira de Abreu et al., 2007). The resulting materials, which are frequently polymers with nanoparticles incorporated in their structure, present improvements of barrier properties, such as thermal, mechanical or permeability characteristics, providing innovative solutions to increase the performance of the polymers (Silvestre et al., 2011). Therefore, nanotechnology plays an important role in the preservation of perishable food.

Among the most commonly used materials in food packaging are nanocomposites based on organic polymers and inorganic clay minerals (Pereira de Abreu et al., 2007). These clays consist of silicate nanoplates whose structure presents platelet morphology. Once the clays have been incorporated into the polymer, the resulting platelets force gases and other

external agents to follow a tortuous path through the material, decreasing and slowing the contact with the product obtaining more resistant plastics (Pereira de Abreu et al., 2007; AESAN, 2010).

The Technological Institute of Packaging, Transport and Logistic (ITENE) is developing modified clays based in montmorillonite (natural phyllosilicate), one of the main mineral clays used to be incorporated in food packaging. The use of functionalized clays, immersed and dispersed in the polymers for packaging results in nanocomposite materials, conformed by platelets with submicrometer dimensions, except for their thickness, which present nanometric dimensions (Hatzigrigoriou et al., 2011). These dispositions allow preventing the exchange of oxygen, carbon dioxide and humidity with the food, maintaining its organoleptic and nutritional characteristics for a longest period of time (Farhang, 2009).

The nanoscale formulation of materials change physicochemical properties compared with the bulk or microstructurated material. Smaller size, higher surface to volume ratio and greater reactivity are properties that confer advantages to the use of nanomaterials. Moreover, the toxicity and toxic kinetic profile also change, since it cannot be inferred from data of their non-nanostructured homologous. Therefore, the potential risk for the health and the environment could be modified (EFSA, 2009), making necessary to investigate the toxicity profile of these modified clays, even more, taking into account the scarce toxicological information available so far.

Considering that the consumer exposure to these nanoclays is likely since they are present in the food packaging, it is interesting to evaluate not only the microstructured clay, but also

the imbued nanomaterial in the polymer, due to the possible migration to the food product (Guillard et al., 2010; EFSA, 2011; Song et al., 2011).

The EU Commission Regulation N° 10/2011 and the Council Directive 82/711/EEC collect the aspects related to plastic materials and articles intended to come in contact with food and foodstuffs, as well as the simulants used in the migration assays, and it states that the risk assessment should cover the potential migration under worst foreseeable conditions of use and the toxicity. Moreover, the Commission Regulation EU nº 10/2011 also establish an overall migration limit of 10 mg/dm^2, this is plastic materials and articles shall not transfer their constituents to food simulants in quantities exceeding 10 milligrams of total constituents released per dm^2 of food contact surface.

As nanoclays are substances that could be potentially present in food, the oral pathway would be the most commonly entry for these substances in the organism. Thus, it would be interesting to establish the toxicological profile of the migration extracts with *in vitro* assays, and thereby contribute to the risk assessment of nanomaterials used in food industry, claimed by the European Food Safety Authority (EFSA, 2011). In this sense, the present study provides a toxicological evaluation of two migration extracts obtained from nanocomposite materials with PLA and two modified clays developed by ITENE, Clay1 and Clay2, through several cytotoxicity assays on two specific and target cell lines (Caco-2 and HepG2 cells, from intestinal and liver origin, respectively) and a mutagenicity assay, the Ames test.

Moreover, the presence of characteristic metallic elements of the clays structure (Al, Ca, Fe, Mg, Si) was also determined in the migration extracts.

2. Materials and Methods

2.1. Supplies and Chemicals

Minimal Essential Medium (MEM) powder (1x) with Earle´s Salts was obtained from PPA laboratories GmbH (Austria) and other cell culture reagents were supplied from Gibco (New Zealand). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Cell Culture

Caco-2 cell line derived from human colon carcinoma (ATCC Nº HTB-37) was maintained at 37ºC in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire, Spain), in a medium consisting of Minimum Essential Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 mg/mL gentamicin, 1.25 mg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. The cells were used at passages between 12-21. HepG2 derived from a liver hepatocellular carcinoma (ATCC Nº HB-8065) was maintained at similar conditions in EMEM supplemented with 10% FBS, 100 U/mL penicillin and 100µg/mL streptomycin. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested each two-three days with 0.25% trypsin. The cells passes used were 16-32.

2.3. Clays and polymer

Purified sodium montmorillonite (MMT) (Cloisite® Na^+), with cationic exchange capacity (CEC) of 116meq/100g was purchased in Southern Clay Products (COMITEX, S.A. Spain).

Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) ($C_{19}H_{42}BrN$, 364.46 g/mol, 98%) was supplied from CymitQuímica S.L. (Spain), acetylcholine chloride (ACO) ($C_7H_{16}ClNO_2$, 181.66 g/mol, ≥99%) was provided by Sigma-Aldrich (Spain). Polylactide (PLA) pellets were purchased from Cargill Dow (NatureWorks ® PLA 7032 polymer) for the preparation of filled biopolymers (Fig.1).

2.4. Organo-modified nanoclays

Organo- modified clays have been developed and characterized by ITENE based on previous works (Jordá Beneyto et al., 2008; Jordá et al., 2009). Briefly, two different organo-modified clays were prepared by a cation-exchange method, which consists on a displacement of the sodium cations of Cloisite® Na^+ with the ammonium cations of the above mentioned salts, obtaining Clay1 and Clay2. Both clays were characterized by Fourier Transform InfraRed (FTIR), Wide-angle X-ray Diffraction and Termogravimetric Analysis (TG) following the methods described in Jordá- Beneyto et al. (2013).

2.5. Nanocomposites preparation

Different PLA nanocomposites samples have been obtained with the prepared modified clays, Clay1 (MMT + HDTA) and Clay 2 (MMT + HDTA + ACO) with 4 % clay content. For this purpose a twin screw extruder DSE 20/40 (COPERION) was used. The twin screw extruder was used to prepare the samples through melt- direct intercalation. PLA pellets (dried overnight at 90ºC and vacuum for 2 hours (MANN HUMMEL PicolinoDehumidifier)) were blended with 4% in weight of Clay1 and Clay2, respectively. Bottles were obtained by injection stretch blow moulding (ISBM). An important part of the process is the mechanical

stretching of the preform during the moulding process, which helps to increase the impact resistance of the container and also helps to produce a very thin walled container, affecting final mechanical, thermal and chemical properties of the nanocomposite material. The thermal, mechanical and barrier properties of the materials obtained were analyzed following the techniques described in Jordá-Beneyto et al. (2013).

2.6. Migration Extracts

Migrations extracts of both nanocomposites were provided by ITENE. Migration tests were carried out according to UNE-EN 1186-9:2002 (AENOR, 2002). The samples, bottles of 150 mL of nanocomposite materials PLA+ Clay 1 and PLA+ Clay 2, (123,655 dm^2 and 590-598 µm thick) were filled with the simulant chosen according to the law (EU Commission Regulation N° 10/2011 and Council Directive 82/711/EEC), distilled water, at the specific conditions established by the regulations (Temperature(C°): 40; Time (days): 10). Then, all volume of simulants were poured into cell glasses and exposed to heat in order to the simulants were evaporated. Afterwards, the residues were weighed and compared with control cells glasses (without simulants) and the presented difference is the global migration from the simulants. Moreover, nanocomposites of PLA -Clay1, and, PLA -Clay2, were also filled with the specific culture medium of each cell line used as simulant. Each sample was evaluated by triplicate.

2.7. Analysis of Metal Content in the Migration Extracts

The presence of characteristic metals of the clays structure in PLA-Clay1, PLA-Clay2 and only PLA (as control) unfiltered extracts was analyzed. Al, Fe and Mg were quantified by (ICP-MS) (Agilent 7500C, Agilent ICP-MS Systems, USA). Also, Si and Ca were studied by (ICP-OES)

(Horiba Jobin Yvon 2). These determinations were carried out in the Microanalysis service of the Research, Technology and Innovation Center of University of Seville (CITIUS) based on standard operating protocols.

2.8. Cytotoxicity assays

Caco-2 and HepG2 cells were seeded in a 96-well tissue-culture plates at 7·x 10^5 cells/mL, and incubated at 37°C for 24 h prior to exposure. Cells were exposed for 24 and 48h to the following concentrations: 2.5, 5, 10, 20, 40, 60, 80 and 100% of the migration extracts of PLA-Clay1 and PLA-Clay2 reconstituted with powder culture medium. Exposures were also performed with culture medium used as simulant in the migration tests. The basal cytotoxicity biomarkers assayed were: tetrazolium salt reduction (MTS), neutral red cellular uptake (NR) and protein content (PC).

MTS reduction is carried out by dehydrogenases enzymes present in mitochondria. MTS reduction was measured in intact cells according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt) tetrazolium compound added to the medium is bioreduced by cells into a colored formazan product soluble in culture medium which is directly measured with the spectrophotometer at 490 nm after 3 h of incubation in the dark. The lysosomal uptake neutral red (NR) was performed according to Borenfreund and Puerner (1984). The absorbance of the extracted NR from the viable cells was measured in a spectrophotometer at 540 nm. Protein content (PC) was quantified in situ, according to the procedure given by Bradford (1976). The culture medium was replaced by 200 mL NaOH and after 2 h incubation

at 37 ºC, 180 µL was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature absorbance was read at 620 nm.

2.9. Ames test

The incorporation version of the Ames test was performed according to the recommendations of Maron and Ames (1983) and following the principles of OCDE guideline 471 (1997). Five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used for the assay. Cultures of each tester strain were prepared from their main strain plates, and use in late exponential growth phase. Each test substance was assessed in three independent experiments, each conducted in absence and presence of S9 metabolic activation system from rats livers (Aroclor 1254, Sigma, Spain), using triplicate plates for each test substance concentration. Both extracts were tested in five different concentrations (20%, 40%, 60%, 80%, 100%). Briefly, 0.1mL of test substance, 0.1mL of bacterial culture and 0.5mL of S9 mix, were added to 2mL molten agar at 42° and poured onto Vogel Bonner-E minimal glucose agar plates. Plates were inverted and incubated 65-70 h at 37°C in the dark. Revertant colonies were counted and background lawn was inspected for signs of toxicity or compound precipitation. 2-Nitrofluorene (2-NF) (0.1 µg/plate) and sodium azide (NaN_3) (1 µg/plate) were selected as positive controls.

2.10. Calculations and statistical analysis

All experiments were performed three times and at least in triplicate per concentration. Data of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control group, being unexposed cells. Statistical analysis was

carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests using GraphPadInStat software (GraphPadSoftware Inc., La Jolla, USA). The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlett´s test, respectively. Differences were considered significant from $p< 0.05$.

3. Results

3.1. Migration Extracts of PLA-Clay1 and PLA-Clay2.

Overall migration tests were carried out in 150 mL bottles of nanocomposite materials (123.655104 cm^2 total area) according to UNE-EN 1186-9 standard (2002). The results showed values of 0.1 ± 0.2 mg/dm^2 for each sample.

3.2. Metal Content on the Migration Extracts

Aluminum contents of the control and PLA-Clay2 extract were lower than the limit of detection (LOD) and quantitation (LOQ) of the technique, respectively (Table 1). However PLA-Clay1 extract showed 0.7± 0.2 µg/L Al. Ca contents could not be quantified in any sample. Similarly, Fe contents were ≤ LOD in all the samples. Mg contents in PLA-Clay1 showed the highest value in comparison to PLA and PLA-Clay2 extracts. Finally, Si was the most abundant element, showing similar contents in PLA and PLA-Clay1, but lower content was presented in PLA-Clay2. Overall, the metal levels found were very low and always under the currently values permitted by the European Union (Commission Regulation EU nº 10/2011).

3.3. Basal Cytotoxicity assays

3.3.1. Migration Extract of PLA-Clay1 in Caco-2 and HepG2 cell lines.

The migration extract of PLA-Clay1 showed no cytotoxic effects on Caco-2 cells in the three biomarkers assayed. In the MTS assay, no significant differences with respect the control were observed in any concentration assayed after 24 and 48 h exposure (Fig. 2A). Similar results were obtained with neutral red uptake (Fig. 2B); no significant differences were observed from 0% to 100% after 24 and 48 h of exposure. Regarding protein content, the same pattern was followed in this endpoint; however, lower results were observed after 48h of exposure respect to 24h (Fig.2C).

The results obtained in the MTS, NR and PC assays for the HepG2 cell line exposed to the migration extract of PLA-Clay1 did not show significant differences with respect to control group in any of the concentrations assayed after 24 and 48 h of exposure (data not shown).

3.3.2. Migration extract of PLA-Clay2 in Caco-2 and HepG2 cell lines.

When Caco-2 cells were exposed to the migration extract of PLA-Clay2 similar results were observed to those obtained with the migration extract of PLA-Clay1 in the same cell line. No evidence of cytotoxicity was observed in any of the biomarkers assayed (Fig. 3).

The results observed when HepG2 cells were exposed to the migration extracts of PLA-Clay2 did not show significant differences at the range of concentrations assayed after 24 and 48 h of exposure in the MTS metabolization, NR uptake and PC assay(data not shown).

The absence of cytotoxic effects was also observed when cells were exposed to the cell culture medium used as simulant.

3.4. Ames test

After exposure to PLA-Clay1 and PLA-Clay2 there was no increase in the number of revertants and no indication of mutagenic activity in any of the treated strains up to the highest concentration (100%) of the extracts, either in the presence or absence of S9 (Table 2). Toxicity, measured as a decrease in background lawn and in revertant frequency, was not observed at any concentration tested. Positive controls produced statistically significant increases (p<0.01) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix. It was concluded that both extracts were not genotoxic in this bacterial assay.

4. Discussion

There is a major concern about food safety of packaging materials relative to the possible migration of packaging constituents, even more when the packaging is produced by a novel technology. In the present research work, in order to evaluate novel nanocomposite materials intended to be used in food packaging, several *in vitro* toxicity studies have been performed with their migration extracts.

The results obtained in the migration tests with the two nanocomposite materials employed are the first reported for these compounds since they have been developed by our research group and they are not yet commercialized. The values obtained are below the migration limits required by the legislation (Commission Regulation (UE) Nº 10/2011), which establishes that the plastic materials should not release more than 10 mg of substance per 1 dm^2 of the plastic surface. Other researchers have evaluated several modified polymers with other different modified clays, including in some cases MMT. Thus, Schmidt et al. (2012),

Busolo et al. (2012) or Avella et al. (2005) also found migration levels in accordance with the EU legislation. Nevertheless, Commission Regulation (UE) Nº 10/2011 establishes that these substances (nanoparticles) should be assessed on a case-by-case basis as regards their risk until more information is known about such new technology.

Cytotoxicity assays on Caco-2 and HepG2 cells exposed to migration extracts of the nanocomposite materials showed similar results in both cases. No significant differences were observed in the range of concentrations assayed, and total cell viability was unaltered in all cases. These results confirm that the migration extracts of the nanocomposite materials containing Clay1 and Clay2 are not cytotoxic at the conditions assayed. This could be considered an important start-point for the potential commercial launch of these nanocomposites for food packaging applications considering that consumer's safety is requested.

Scarce studies have reported so far regarding to cytotoxic effects induced by extracts of nanocomposite materials and modified montmorillonite clays. Rodrigues da Silva et al. (2010) observed the biocompatibility and cytotoxicity of the biodegradation products of two modified polyurethanes (PUD) intended to medical applications; PUD5 that contains poly (caprolactone) as soft segment, and PUD6, which has poly (caprolactone) and poly (ethylene glycol) as soft segment, with montomorillonite. After 48h, at 3:10 ratio, the cell viability of the human retinal pigment epithelial cell (ARPE-19), measured by mitochondrial conversion of the tetrazolium salt, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), decreased in the modified polyurethanes and nanocomposites supplemented mediums (94,2 ± 3,26 %), although the statistical analysis showed no significant differences on the viability

of ARPE-19 cells cultures in the PUD supplemented and control mediums. Fukushima et al. (2012) also studied the cytotoxicity of the degradation products of poly(butylenesadipate-co-terephthalate)(PBAT) modified with modified and unmodified montmorillonites, and other two clays, modified and unmodified fluorohectorites and unmodified sepiolite (intended to medical and industrial applications). This research group evaluated the biocompatibility of PBTA and nanocomposites with 10% of clay content trough an *in vitro* cytotoxicity and a protein adsorption test, chosen as experiment model fibroblast 929 cells. Cell viability and morphology were measured using MTT assay and inverted microscopy, respectively, after 0, 24, 72 and 120 h of incubation with a dilution of each extract. MTT results revealed no visible reduction in viability between the negative control and experimental groups at all times assayed. At the same time, PBAT and nanocomposites showed similar increases in the relative cell growth depending on the time of incubation, evaluated by direct observation from micrographs, evidencing that the addition of the nanoparticles did not induced further cytotoxic effects in L929 cells. In addition, the cytotoxicity of degradation products of chitin based polyurethane bio-nanocomposites (PUBNC), intended to food packaging, and prepared with bentonite nanoclay enriched in montmorillonite (MMT) and other compounds have been investigated by Zia et al. (2011). The cytotoxicity was measured by live/dead staining of L-929 fibroblast cells exposed to different concentrations of degradation products of the nanocomposite material, 0, 1, 2, 4 and 8% clay content showing significant differences in the groups containing 2-8% of bentonite nanoclay with respect to control and 1% bentonite content group.

Interestingly, the cytotoxicity results obtained with the migration extracts from nanocomposites containing Clay1 and Clay2 differs from the *in vitro* evaluation of the initial microstructured clays. In previous studies carried out in our laboratory, no cytotoxic effects were observed in HepG2 cells exposed to Clay1 from 0 to 8 µg/mL (no higher concentrations could be tested due to its hydrophobicity) after 24 and 48 h (Houtman et al., 2013). However, our research group observed significant cytotoxicity effects on HepG2 exposed to Clay2, in a range of 0 to 125 µg/mL during 24 and 48h, obtaining mean effective concentration (EC_{50}) values for the MTS assay of 88 and 51 µg/mL, respectively (Houtman et al.,2013, Jordá-Beneyto et al., 2013). Similarly, no effect was recorded in Caco-2 cells exposed to Clay1, although in the case of Clay2, EC50 values of 34 and 18.5 µg/mL after 24 and 48h, respectively were found for the PC. Overall, Clay1 did not show cytotoxic effects in any case, whereas Clay2 in its microstructured formulation seems to be toxic on HepG2 and Caco-2 cells; but no toxicity was found once the clay was imbued in the polymer.

A test for induction of gene mutations in bacteria is among the core set of toxicological tests required in the Guidelines of the Scientific Committee on Food for the presentation of an application for safety assessment of a substance to be used in food contact materials prior to its authorization (European Commission 2001). In the present study any of the extracts have shown mutagenic potential with and without metabolic activation. Sharma et al. (2010) also observed no mutagenic activity in TA98 and TA100 exposed to filtered and unfiltered suspensions of purified sodium MMT (Cloisite® Na^+) and the commercial organoclay Cloisite® 30B. Also, Li et al. (2010) evaluated the mutagenic potential of exfoliated silicate nanoclay in

TA98, TA100, TA1535, TA1537 and TA102. In this case, revertants colonies were not present in any strain assayed compared to the negative control plates, in presence or absence of S9. The absence of a toxic response in this test is in accordance with the results obtained in the cytotoxicity studies.

Regarding the study about the presence of metals in the migration extracts, no significant differences were observed in the analysis. However, Avella et al. (2005) studied the metals migration from nanocomposites films with potato starch and montmorillonite to lettuce and spinach. No differences were observed in Fe and Mg content in the vegetables, while a higher Si content was observed. This finding could be easily attributable to the fact that clay nanoparticles are mainly composed of this element. On the other hand, Busolo et al. (2012) investigated the migration of Al and Fe from polyolefin nanocomposite films containing an iron modified kaolinite with two simulants, water and isooctane, and, no differences were observed in any samples assayed.

5. Conclusion

The global migration obtained for the nanocomposites assayed was below the legislation recommendations, as well as their metallic content. Moreover, no cytotoxic and genotoxic effects were observed with the migrations extracts.Further toxicological studies are needed to confirm the safety of these nanocomposites for their use in food packaging applications.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, the Cell Culture Service and Microanalysis Service of Centro de Investigación and Tecnología e Innovación de la Universidad de Sevilla (CITIUS), Consuelo Fernández Rivas and Jose María Bermúdez for providing technical assistance.

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Table captions

Table 1. Al, Ca, Fe, Mg and Si content in the migration extracts from PLA, PLA-Clay1 and PLA-Clay2 bottles (all the concentrations are expressed in μg/L).

Table 2. Results of Ames test conducted with PLA-Clay1 and PLA-Clay2 extracts for three independent experiments. Water was used as negative control and DMSO as solvent for positive controls. Data are given as mean±SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/ TA98/ TA102/ TA104 with and without S9: 2-NF (0.1 μg/plate) and TA100 with and without S9: NaN_3 (1μg/plate). ***p*<0.01 significantly different from control.

Figure captions

Figure 1. Structure of the quaternary ammonium salts: Acetylcholine chloride (a) and Hexadecyltrimethylammonium bromide (b) used to modify the montmorillonite.

Figure 2. Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in Caco-2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay1 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).

Figure 3. Cytotoxicity assays: MTS metabolism (A), neutral red uptake (B) and total protein content (C) in Caco-2 cells after 24 and 48 h of exposure to 0 – 100% PLA-Clay2 migration extract (n=3). All values are expressed as mean ± SD. *Significantly different from control ($p \leq 0.05$).

Table 1

Mean ± SD (µg/L)					
	Al	Ca	Fe	Mg	Si
PLA	≤LOD	≤LOD-≤LOQ	≤LOD	≤LOD-2.1	28.5±1.5
PLA-Clay1	0.7±0.2	≤LOD	≤LOD	3.6±0.7	29.1±1.1
PLA-Clay2	≤LOQ	≤LOD	≤LOD	1.51±0.1	20.5±3.5

LOD for Al, Ca, Fe, Mg and Si were 0.2, 42, 0.3, 0.4, 5 µg/L, respectively.
LOQ for Al, Ca, Fe, Mg and Si were 0.5, 140, 0.6, 1, 14 µg/L, respectively.

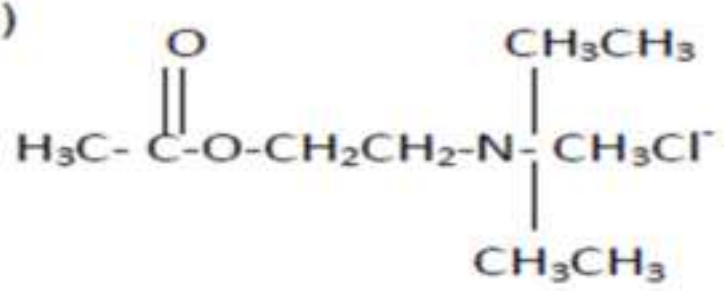
Table 2

	Concentration (%)	TA97A		TA98		TA100		TA102		TA104	
		+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
PLA-Clay 1 Extract	Negative controls	402±18	338±54	20±1	21±4	123±34	115±25	246±65	246±40	326±26	320±59
	20%	286±67	368±2	22±6	23±9	104±35	100±26	236±30	221±92	320±99	349±7
	40%	438±10	409±65	24±5	21±3	101±40	109±19	352±62	324±76	418±40	409±65
	60%	424±11	320±11	32±2	27±4	122±1	124±20	365±57	353±120	284±22	307±16
	80%	357±22	343±42	25±1	18±1	96±36	107±29	297±47	256±9	256±42	295±61
	100%	342±34	271±49	30±5	21±3	143±1	154±8	260±8	272±53	286±40	266±41
	Positive controls	722±28**	751±24**	>1000**	>1000**	>1000**	>1000**	853±174**	621±172**	>1000**	884±79**
PLA-Clay 2 Extract	Negative controls	344±47	398±17	41±5	26±18	125±33	115±19	223±23	244±46	315±41	297±44
	20%	342±5	299±24	47±13	27±7	109±20	106±10	296±9	291±21	317±3	293±46
	40%	396±34	317±47	37±1	19±6	103±8	116±62	265±54	271±35	334±27	272±31
	60%	393±12	323±27	38±8	26±8	134±25	112±25	294±17	277±46	341±33	308±34
	80%	285±49	301±3	26±1	29±11	96±29	96±14	324±178	281±73	297±12	283±14
	100%	331±19	340±37	31±18	24±4	110±40	96±15	279±2	264±33	289±46	248±46
	Positive controls	650±13**	754±6**	>1000**	>1000**	>1000**	>1000**	853±175**	722±30**	>1000**	917±32**

Figure 1

Click here to download high resolution image

a)



b)

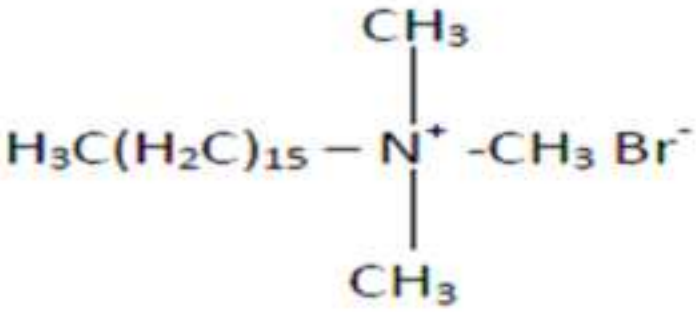
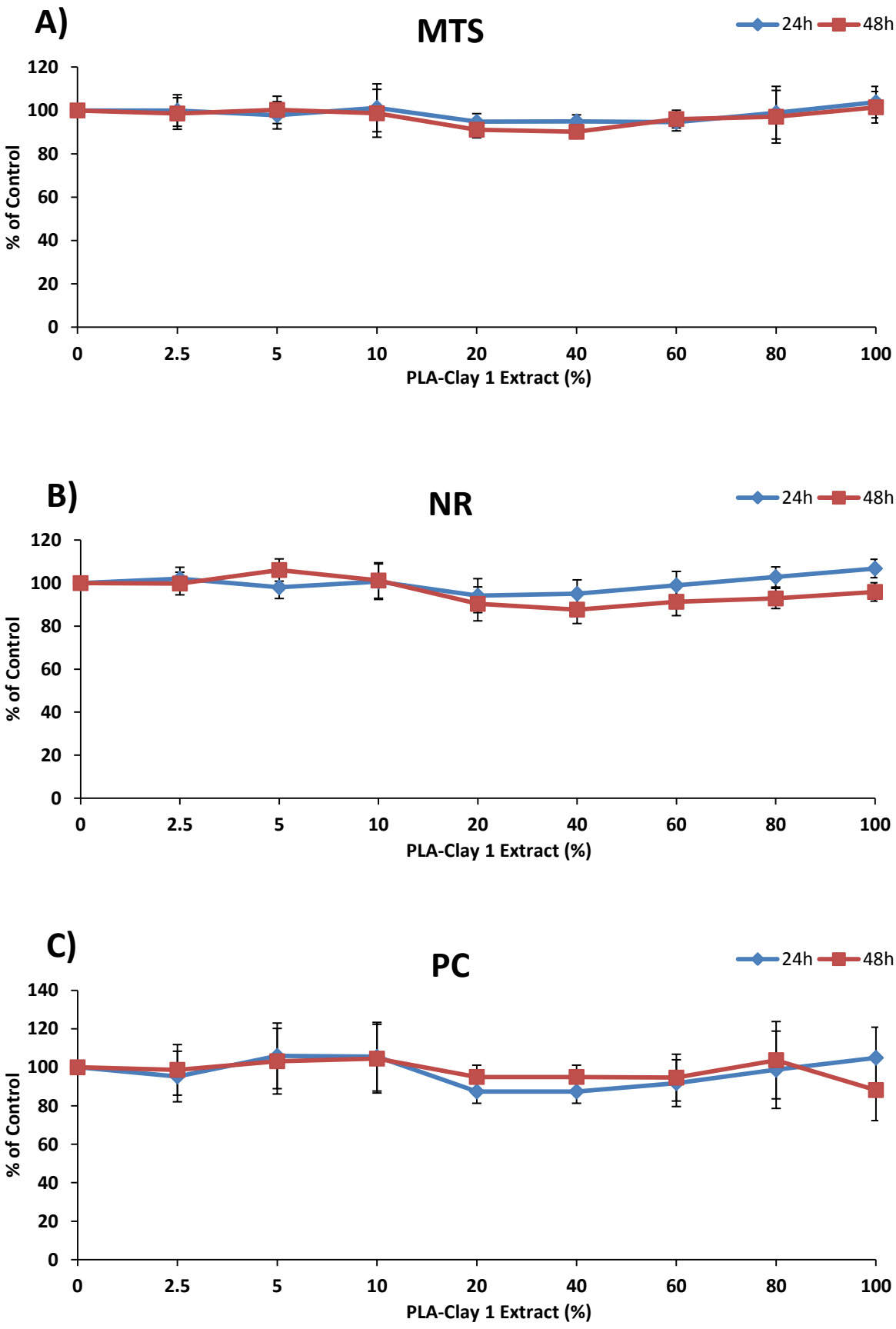


Figure 2


A)
MTS
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% of Control
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PLA-Clay 1 Extract (%)
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PLA-Clay 1 Extract (%)
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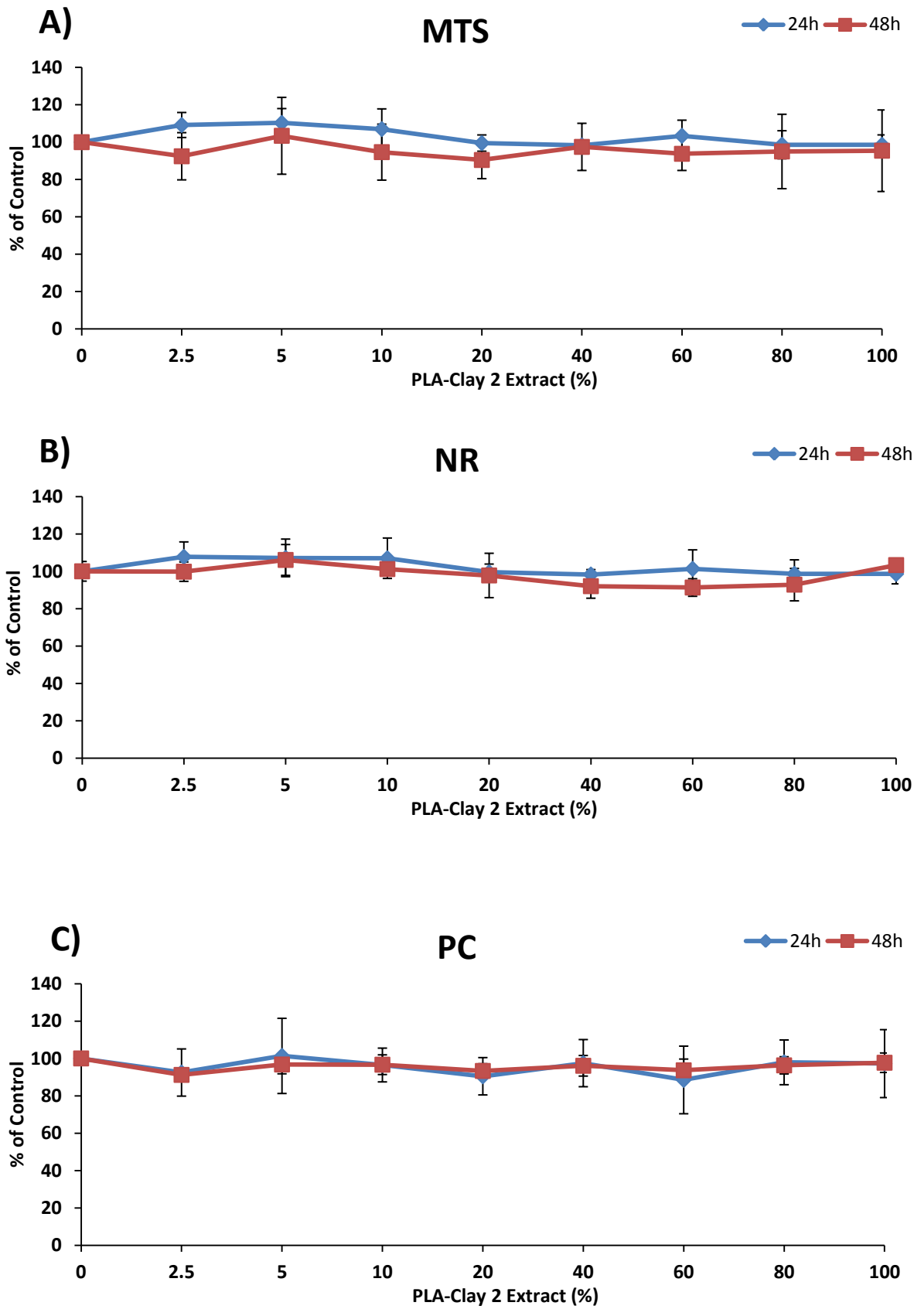
PLA-Clay 1 Extract (%)

Figure 3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PLA-Clay 2 Extract (%)
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PLA-Clay 2 Extract (%)
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2.5
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100
PLA-Clay 2 Extract (%)

CAPÍTULO 10 / CHAPTER 10

Sara Maisanaba, Daniel Gutiérrez-Praena, María Puerto, Rosario Moyano, Alfonso Blanco, María Jordá, Ana M. Cameán, Susana Aucejo, Ángeles Jos

EFFECTS OF THE SUBCHRONIC EXPOSURE TO AN ORGANOMODIFIED CLAY MINERAL FOR FOOD PACKAGING APPLICATIONS ON WISTAR RATS

Applied Clay Science 95, 37-40, ***2014***

Elsevier Editorial System(tm) for Applied Clay Science

Manuscript Draft

Manuscript Number: CLAY5809R2

Title: Effects of the subchronic exposure to an organomodified clay mineral for food packaging applications on Wistar rats

Article Type: Note

Keywords: clay mineral; montmorillonite; sub-chronic toxicity; rat; histopathology

Corresponding Author: Dr. Angeles Jos,

Corresponding Author's Institution: University of Sevilla

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Daniel Gutiérrez-Praena; María Puerto; Rosario Moyano; Alfonso Blanco; María Jordá; Ana M Cameán; Susana Aucejo; Angeles Jos

Abstract: Organomodified clay minerals have many present and potential applications in different fields, including food packaging. For these applications become a reality they need a successful technological development but also they have to demonstrate their safety. There are in vitro toxicological studies that have shown a reduction of viability and oxidative stress induced by organomodified clay minerals in human cell lines. However, in vivo toxicity reports are scarce, particularly those using the oral pathway. The aim of this work was to evaluate the toxicity of an organomodified clay mineral, Clay1, developed from montmorillonite (Mt) and using hexadecyltrimethylammonium bromide as modifier. A repeated dose 90-day oral toxicity study was performed in Wistar rats exposed to 40 mg/kg/day Clay1. The morphological study of the main organ tissues by optic and electronic microscopy did not reveal any adverse effect. Also, blood clinical biochemistry parameters, the reduced/oxidized glutathione (GSH/GSSG) ratio, and interleukin-6 leakage in serum did not show any significant alterations. These results suggest that Clay1 do not cause remarkable toxic effects at the conditions tested.

Suggested Reviewers: Jose M Navas
INIA Instituto Nacional de Tecnología Agraria y Alimentaria
jmnavas@inia.es

Guillermina Font
Universidad de Valencia
Guillermina.Font@uv.es

Nicola Mascolo
Universita Federico II
nicola.mascolo@unina.it

Fabio Tateo
Instituto di Geoscienze e Georisorce. Padua. Italy
tateo@igg.cnr.it

Effects of the subchronic exposure to an organomodified clay mineral for food packaging applications on Wistar rats

Sara Maisanaba[a], Daniel Gutiérrez-Praena[a], María Puerto[a], Rosario Moyano[b], Alfonso Blanco[c], María Jordá[d], Ana M Cameán[a], Susana Aucejo[d], Ángeles Jos[a]*

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[b] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, Faculty of Veterinary, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[c] Department of Anatomy and Comparative Pathology and Anatomy, University of Cordoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[d] Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

*Corresponding author:

Ángeles Jos

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: angelesjos@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Organomodified clay minerals have many present and potential applications in different fields, including food packaging. For these applications become a reality they need a successful technological development but also they have to demonstrate their safety. There are in vitro toxicological studies that have shown a reduction of viability and oxidative stress induced by organomodified clay minerals in human cell lines. However, in vivo toxicity reports are scarce, particularly those using the oral pathway. The aim of this work was to evaluate the toxicity of an organomodified clay mineral, Clay1, developed from montmorillonite (Mt) and using hexadecyltrimethylammonium bromide as modifier. A repeated dose 90-day oral toxicity study was performed in Wistar rats exposed to 40 mg/kg/day Clay1. The morphological study of the main organ tissues by optic and electronic microscopy did not reveal any adverse effect. Also, blood clinical biochemistry parameters, the reduced/oxidized glutathione (GSH/GSSG) ratio, and interleukin-6 leakage in serum did not show any significant alterations. These results suggest that Clay1 do not cause remarkable toxic effects at the conditions tested.

Keywords: clay mineral, montmorillonite, sub-chronic toxicity, rat, histopathology

1. Introduction

Natural clay minerals are widely used in catalysis, as adsorbents, in clay polymer nanocomposites (CPN), as antibacterial materials, nuclear waste storage, pesticide carriers, etc. (Liu, 2007). Particularly food packaging industry has mainly focused its attention on layered inorganic solids, such as clay minerals ~~and silicates~~, due to their availability, low cost, significant enhancements and relative simple processability (De Azeredo, 2009; Hatzigrigoriou et al., 2011). As a result, CPN were the first nanocomposites to be developed and introduced to the market as enhanced materials for the food- packaging sector (Hatzigrigoriou et al., 2011.). The most widely studied ~~type of~~ clay mineral as fillers is montmorillonite (Mt)~~sheets~~ (Weiss et al., 2006).

The main advantage of using clay minerals as fillers is a marked increase in the barrier of the polymer material to gas and water (Silvestre et al., 2011). Other authors also mention the improvements in mechanical, thermal, optical and rheological properties of the CPN (De Azeredo, 2009; Haztigrigoriou et al., 2011), thus increasing the product shelf life.

One limitation of using clay minerals as nanofillers is the incompatibility between the hydrophilic clay mineral and a hydrophobic polymer (Elmore and Andersen, 2003; Zeng et al., 2005). Therefore, chemical modification of clay minerals is an important step to achieve CPN. In this sense by ion exchange with long-chain organic cations, clay minerals become hydrophobic and thereby compatible with

polymers. Such modified clay minerals are referred to as organoclays ~~minerals~~(OC) (Sharma et al., 2010).

Due to the aforementioned wide range of applications ~~organoclay minerals~~OC can have, the human exposure to this kind of compounds is likely going to increase in the near future. This makes necessary a safety evaluation since potential toxic effects cannot be discarded. Toxic effects of clay minerals have been shown to occur mainly after inhalation (Carretero et al., 2006; Sharma et al., 2010), but the oral pathway is one of the most likely routes of exposure to these clay minerals for the general population, since they are present in food contact materials.

Although toxicological information of clay minerals is scarce, different authors have reported toxic effects on in vitro systems, such as reduced viability or reactive oxygen species production (Lordan et al., 2011) Houtman et al. (2014) and Maisanaba et al. (2013a,b), have found a different toxic profile of different modified and unmodified clay minerals in Caco-2 and Hep-G2 cells and have suggested that a case by case toxicological assessment is required for clay minerals, as the modifiers employed to improve their technological aspects play an important role in the toxicity observed.

In vivo toxicity data of clay minerals are even scarcer, and most of the trials performed are not representative of the likely human exposure to these compounds, which corresponds to a repeated and low dose exposure for a long time. Taking all this into account, the aim of this study was to determine the oral subchronic toxicity of an ~~organoclay mineral~~OC on Wistar rats. A morphological study of the main organ tissues by optic and electronic microscopy was performed. Blood clinical biochemistry

parameters, interleukin leakage in serum and blood glutathione levels were also determined.

2. Materials and Methods

2.1. Supplies and Chemicals

General chemicals were provided by Sigma Aldrich (Madrid, Spain) and VWR International Eurolab (Spain).

The organomodified clay mineral encoded as Clay1 was obtained by cation exchange reaction from Cloisite Na^+® (Southern Clay Products, INC.), adding the quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) in 6 fold the cation exchange capacity (CEC) of pristine clay mineral, following the method described by Mittal (2007), Jordá- Beneyto et al., (2008) and Jorda et al., (2009). The modified clay mineral was characterized by Fourier Transform Infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA) and X-ray diffraction (XRD) as shown in Jordá-Beneyto et al., (2014).

2.2. Animals and Experimental Design

A repeated dose 90-day oral toxicity study was performed in rats. Briefly, twenty male Wistar rats provided by Janvier S.A.S (France) with a mean weight of 240.2 ± 2.04 g were used. Rats were fed during the acclimation week with a standard laboratory diet (Harlan 2014, Harlan Laboratories, Barcelona, Spain), and water, both *ad libitum*, in a controlled-temperature room (23 ± 1ºC) with 12 h dark/light cycle, and free from any source of chemical contamination. After this period, animals were randomly divided in two groups, a control group (n=10) and an exposed group (n=10).

The control group was fed with the standard diet, while the exposed group was orally administered with 40 mg/kg/day of Clay1 in the diet. This dose was selected to reproduce 2-fold the dose that a person would received daily from a 1.5L bottle made of the CPN. This is, assuming that all the clay mineral incorporated into the packaging polymer is present in the food. During the treatment period clinical signs, body weight, and food and water consumption were recorded weekly.

All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes and all the procedures were previously accepted by the Ethic Committee of the University of Seville.

2.3. Organs and blood sampling

At the end of the experimental period, rats were fasted for 18 h before sacrifice. Liver, kidneys, lungs, spleen, brain, testes, gastrointestinal (g.i.) tract and heart were excised, rinsed with cold saline solution and weighted. Blood samples were obtained by cardiac punction and collected in test tubes with/without lithium heparin depending on the experiment. Serum was separated by low speed centrifugation at 1500 x g at 4ºC for 15 min, and stored at -80ºC until analysis of IL-6 leakage and clinical biochemistry parameters.

2.4. Histopathogical analysis

The histopatological examination by optic (Hematoxylin-Eosin HE staining) and electronic microscopy was performed as described by Maisanaba et al. (2014).

2.5. GSH/GSSG levels

The ratio GSH/GSSG was determined in blood samples using a commercial kit (Bioxytech GSH/GSSG- 412; Oxis Research, Foster City, CA, USA).

2.6. IL-6 leackage

For this assay the blood serum of experimental animals were used. Manufacturer instructions from the kit (Thermo Scientific Rat Interleukin-6 (IL-6) ELISA) were followed.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation of ten animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

3. Results

No rats died during the experimental period and there were no remarkable clinical signs. Body weight, body weight gain, food and water consumption , organs weight and the somatic index of the different organs (Table 1) did not show statistical differences between the control and the exposed group.

3.1. Histopathogical results

Microscopic examination of the HE-stained tissue sections of the exposed animals did not show remarkable changes in comparison to the control group (Fig. 1). Moreover, the ultrastructural study did not reveal alterations related with the treatment (Fig. 2).

3.2. Clinic Biochemistry Parameters

Results showed no significant changes in any of them in comparison to the control group (Table 2).

3.3. GSH/GSSG levels

Blood GSH/GSSG ratio experienced a non significant alteration in rats exposed to Clay1 in comparison to the control group (Fig. 3).

3.4. IL-6 leackage

No IL-6 leakage was observed in the serum of any of the experimental groups (data not shown).

4. Discussion

Clay1 has been developed to reinforce food packaging materials, thus being considered as a food contact material (Jordá-Beneyto et al., 2014). Human exposure to chemicals from packaging and other materials in contact with food may occur as a result of migration from the packaging materials into foodstuffs. The extent of this migration and the specific toxicity of the substance in question are the two main factors which define the human health risk which a packaging material represents (Pocas and Hogg, 2007). Regarding to its toxicity, Houtman et al., (2014) evaluated the cytotoxic effects of Clay1 in the human intestinal cell line Caco-2 and in the hepatocellular cell line HepG2 and observed a very low toxicity in the range of concentrations assayed (0-8 µg/mL)

To the best of our knowledge, there is no other in vivo toxicology report available of Clay1. In the present study, the results have shown that there are no remarkable toxic effects in the rats exposed for 90 days to Clay1. Baek et al. (2012) performed an acute oral toxicity test in mice with Mt and did not found adverse effects. Similar results were obtained by Li et al. (2010) in rats exposed to exfoliated clay mineral layers~~exfoliated silicate plates~~ prepared from the natural clay mineral. Lee et al., (2005) on the contrary, observed a significant reduction of Na^{+}, Cl^{-} and Ca^{2+} in the blood-biochemical analysis. In these studies a single oral dose was used. Afriyie-Gyawu et al., (2005) performed a 28 week feeding study with NovaSil (calcium Mt) and suggested that levels of 2% w/w did not result in overt toxicity. The absence of histopathological effects of these studies contrast with the morphological adverse effects reported for other clay minerals in vitro (Banin and Mairi, 1990; Elmore et al., 2003; Maisanaba et al., 2013a,b). However, those studies were performed with clay minerals different to Clay1 and the modifiers contained in the ~~organoclay mineral~~OC have a role in their toxicity.

Mascolo et al. (2004) also performed an in vivo assay with several clay minerals but they focused on a different aspect of clay mineral ingestion, the distribution of hazardous chemical elements into organs. Mascolo et al. (1999) documented this fact analyzing the urine of rats fed with different clay minerals and concluded that the chemical elements entered in blood dealing with possible hazardous effects on organs and/or the whole body.

Powell et al. (2007) reported that the human oral exposure to exogenous microparticles appeared to be almost exclusively additives to food, pharmaceuticals and

toothpaste and that they were chiefly aluminosilicates and titanium dioxide. These particles are very resistant to chemical breakdown so, following ingestion and mucosal uptake, they survive both gastrointestinal digestion and cellular processing. Authors hypothesized that exogenous microparticles hijack the putative route for endogenous microparticle uptake but, due to their chemically-resistant nature, they cannot then contribute to tolerance induction and may even, in susceptible individuals, establish abnormal immune responsiveness. In the present study IL-6 in serum has been not detected but a single parameter is not enough to conclude the absence of effects on the immune system.

It has been reported that hemolytic activity of silicate minerals decreases in the order smectites>silica>palygorskite ~~E~~ ≈sepiolite>chrysotile>kaolinite (Oscarson et al., 1986; Carretero et al., 2006~~; Oscarson et al., 1986~~). Clay1 is based on Mt, a clay mineral of the smectites family, but in this case no hemolytic activity and no changes in the clinical biochemistry parameters of the animals after the experimental period has been detected.

5. Conclusion

The morphologic study performed on the tissues of Wistar rats exposed to 40 mg/kg/day Clay1 for 90 days did not evidence any remarkable effect. Similarly, the basic clinical biochemistry parameters, the GSH/GSSG ratio and the IL-6 leakage in serum did not show significant alterations.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, and the Clinical Biochemistry Unit of the University Hospital Virgen Macarena for the analysis performed on serum samples.

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Figure captions

Figure 1. Histopathological changes of Wistar rats exposed to 40 mg/kg/day Clay1 for 90 days. HE-stained sections of liver, kidney, lung, spleen, brain, testes, intestine and heart.

Figure 2. Histopathological changes of Wistar rats exposed to 40 mg/kg/day Clay1 for 90 days. Ultrastructural observations of liver, kidney, lung, spleen, brain, testes, intestine and heart.

Figure 3. Blood GSH/GSSG ratio in control Wistar rats and rats exposed to 40 mg/kg/day Clay1 for 90 days. Results are expressed as mean ± s.d.

Highlights (for review)

- 40 mg/kg/day Clay 1 did not induce subchronic oral toxicity in Wistar rats

- the histopathological study did not reveal remarkable effects induced by Clay1 at the conditions assayed

- clinic biochemistry parameters, glutathione content and IL-6 leakage were not altered by the exposure to Clay1

Effects of the subchronic exposure to an organomodified clay mineral for food packaging applications on Wistar rats

Sara Maisanaba[a], Daniel Gutiérrez-Praena[a], María Puerto[a], Rosario Moyano[b], Alfonso Blanco[c], María Jordá[d], Ana M Cameán[a], Susana Aucejo[d], Ángeles Jos[a]*

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

[b] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, Faculty of Veterinary, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[c] Department of Anatomy and Comparative Pathology and Anatomy, University of Cordoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[d] Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

*Corresponding author:

Ángeles Jos

Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: angelesjos@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Organomodified clay minerals have many present and potential applications in different fields, including food packaging. For these applications become a reality they need a successful technological development but also they have to demonstrate their safety. There are in vitro toxicological studies that have shown a reduction of viability and oxidative stress induced by organomodified clay minerals in human cell lines. However, in vivo toxicity reports are scarce, particularly those using the oral pathway. The aim of this work was to evaluate the toxicity of an organomodified clay mineral, Clay1, developed from montmorillonite (Mt) and using hexadecyltrimethylammonium bromide as modifier. A repeated dose 90-day oral toxicity study was performed in Wistar rats exposed to 40 mg/kg/day Clay1. The morphological study of the main organ tissues by optic and electronic microscopy did not reveal any adverse effect. Also, blood clinical biochemistry parameters, the reduced/oxidized glutathione (GSH/GSSG) ratio, and interleukin-6 leakage in serum did not show any significant alterations. These results suggest that Clay1 do not cause remarkable toxic effects at the conditions tested.

Keywords: clay mineral, montmorillonite, sub-chronic toxicity, rat, histopathology

1. Introduction

Natural clay minerals are widely used in catalysis, as adsorbents, in clay polymer nanocomposites (CPN), as antibacterial materials, nuclear waste storage, pesticide carriers, etc. (Liu, 2007). Particularly food packaging industry has mainly focused its attention on layered inorganic solids, such as clay minerals, due to their availability, low cost, significant enhancements and relative simple processability (De Azeredo, 2009; Hatzigrigoriou et al., 2011). As a result, CPN were the first nanocomposites to be developed and introduced to the market as enhanced materials for the food- packaging sector (Hatzigrigoriou et al., 2011.). The most widely studied clay mineral as fillers is montmorillonite (Mt) (Weiss et al., 2006).

The main advantage of using clay minerals as fillers is a marked increase in the barrier of the polymer material to gas and water (Silvestre et al., 2011). Other authors also mention the improvements in mechanical, thermal, optical and rheological properties of the CPN (De Azeredo, 2009; Haztigrigoriou et al., 2011), thus increasing the product shelf life.

One limitation of using clay minerals as nanofillers is the incompatibility between the hydrophilic clay mineral and a hydrophobic polymer (Elmore and Andersen, 2003; Zeng et al., 2005). Therefore, chemical modification of clay minerals is an important step to achieve CPN. In this sense by ion exchange with long-chain organic cations, clay minerals become hydrophobic and thereby compatible with polymers. Such modified clay minerals are referred to as organoclays (OC) (Sharma et al., 2010).

Due to the aforementioned wide range of applications OC can have, the human exposure to this kind of compounds is likely going to increase in the near future. This makes necessary a safety evaluation since potential toxic effects cannot be discarded. Toxic effects of clay minerals have been shown to occur mainly after inhalation (Carretero et al., 2006; Sharma et al., 2010), but the oral pathway is one of the most likely routes of exposure to these clay minerals for the general population, since they are present in food contact materials.

Although toxicological information of clay minerals is scarce, different authors have reported toxic effects on in vitro systems, such as reduced viability or reactive oxygen species production (Lordan et al., 2011) Houtman et al. (2014) and Maisanaba et al. (2013a,b), have found a different toxic profile of different modified and unmodified clay minerals in Caco-2 and Hep-G2 cells and have suggested that a case by case toxicological assessment is required for clay minerals, as the modifiers employed to improve their technological aspects play an important role in the toxicity observed.

In vivo toxicity data of clay minerals are even scarcer, and most of the trials performed are not representative of the likely human exposure to these compounds, which corresponds to a repeated and low dose exposure for a long time. Taking all this into account, the aim of this study was to determine the oral subchronic toxicity of an OC on Wistar rats. A morphological study of the main organ tissues by optic and electronic microscopy was performed. Blood clinical biochemistry parameters, interleukin leakage in serum and blood glutathione levels were also determined.

2. Materials and Methods

2.1. Supplies and Chemicals

General chemicals were provided by Sigma Aldrich (Madrid, Spain) and VWR International Eurolab (Spain).

The organomodified clay mineral encoded as Clay1 was obtained by cation exchange reaction from Cloisite Na^+® (Southern Clay Products, INC.), adding the quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) in 6 fold the cation exchange capacity (CEC) of pristine clay mineral, following the method described by Mittal (2007), Jordá- Beneyto et al., (2008) and Jorda et al., (2009). The modified clay mineral was characterized by Fourier Transform Infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA) and X-ray diffraction (XRD) as shown in Jordá-Beneyto et al., (2014).

2.2. Animals and Experimental Design

A repeated dose 90-day oral toxicity study was performed in rats. Briefly, twenty male Wistar rats provided by Janvier S.A.S (France) with a mean weight of 240.2 ± 2.04 g were used. Rats were fed during the acclimation week with a standard laboratory diet (Harlan 2014, Harlan Laboratories, Barcelona, Spain), and water, both *ad libitum*, in a controlled-temperature room (23 ± 1ºC) with 12 h dark/light cycle, and free from any source of chemical contamination. After this period, animals were randomly divided in two groups, a control group (n=10) and an exposed group (n=10). The control group was fed with the standard diet, while the exposed group was orally administered with 40 mg/kg/day of Clay1 in the diet. This dose was selected to reproduce 2-fold the dose that a person would received daily from a 1.5L bottle made

of the CPN. This is, assuming that all the clay mineral incorporated into the packaging polymer is present in the food. During the treatment period clinical signs, body weight, and food and water consumption were recorded weekly.

All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes and all the procedures were previously accepted by the Ethic Committee of the University of Seville.

2.3. Organs and blood sampling

At the end of the experimental period, rats were fasted for 18 h before sacrifice. Liver, kidneys, lungs, spleen, brain, testes, gastrointestinal (g.i.) tract and heart were excised, rinsed with cold saline solution and weighted. Blood samples were obtained by cardiac punction and collected in test tubes with/without lithium heparin depending on the experiment. Serum was separated by low speed centrifugation at 1500 x g at 4ºC for 15 min, and stored at -80ºC until analysis of IL-6 leakage and clinical biochemistry parameters.

2.4. Histopathogical analysis

The histopatological examination by optic (Hematoxylin-Eosin HE staining) and electronic microscopy was performed as described by Maisanaba et al. (2014).

2.5. GSH/GSSG levels

The ratio GSH/GSSG was determined in blood samples using a commercial kit (Bioxytech GSH/GSSG- 412; Oxis Research, Foster City, CA, USA).

2.6. IL-6 leackage

For this assay the blood serum of experimental animals were used. Manufacturer instructions from the kit (Thermo Scientific Rat Interleukin-6 (IL-6) ELISA) were followed.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation of ten animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

3. Results

No rats died during the experimental period and there were no remarkable clinical signs. Body weight, body weight gain, food and water consumption , organs weight and the somatic index of the different organs (Table 1) did not show statistical differences between the control and the exposed group.

3.1. Histopathogical results

Microscopic examination of the HE-stained tissue sections of the exposed animals did not show remarkable changes in comparison to the control group (Fig. 1). Moreover, the ultrastructural study did not reveal alterations related with the treatment (Fig. 2).

3.2. Clinic Biochemistry Parameters

Results showed no significant changes in any of them in comparison to the control group (Table 2).

169

170 *3.3. GSH/GSSG levels*

171 Blood GSH/GSSG ratio experienced a non significant alteration in rats exposed
172 to Clay1 in comparison to the control group (Fig. 3).

173

174 *3.4. IL-6 leackage*

175 No IL-6 leakage was observed in the serum of any of the experimental groups
176 (data not shown).

177

178 **4. Discussion**

179 Clay1 has been developed to reinforce food packaging materials, thus being
180 considered as a food contact material (Jordá-Beneyto et al., 2014). Human exposure to
181 chemicals from packaging and other materials in contact with food may occur as a result
182 of migration from the packaging materials into foodstuffs. The extent of this migration
183 and the specific toxicity of the substance in question are the two main factors which
184 define the human health risk which a packaging material represents (Pocas and Hogg,
185 2007). Regarding to its toxicity, Houtman et al., (2014) evaluated the cytotoxic effects
186 of Clay1 in the human intestinal cell line Caco-2 and in the hepatocellular cell line
187 HepG2 and observed a very low toxicity in the range of concentrations assayed (0-8
188 µg/mL)

189

190 To the best of our knowledge, there is no other in vivo toxicology report
191 available of Clay1. In the present study, the results have shown that there are no
192 remarkable toxic effects in the rats exposed for 90 days to Clay1. Baek et al. (2012)
193 performed an acute oral toxicity test in mice with Mt and did not found adverse effects.

Similar results were obtained by Li et al. (2010) in rats exposed to exfoliated clay mineral layers prepared from the natural clay mineral. Lee et al., (2005) on the contrary, observed a significant reduction of Na^{+}, Cl^{-} and Ca^{2+} in the blood-biochemical analysis. In these studies a single oral dose was used. Afriyie-Gyawu et al., (2005) performed a 28 week feeding study with NovaSil (calcium Mt) and suggested that levels of 2% w/w did not result in overt toxicity. The absence of histopathological effects of these studies contrast with the morphological adverse effects reported for other clay minerals in vitro (Banin and Mairi, 1990; Elmore et al., 2003; Maisanaba et al., 2013a,b). However, those studies were performed with clay minerals different to Clay1 and the modifiers contained in the OC have a role in their toxicity.

Mascolo et al. (2004) also performed an in vivo assay with several clay minerals but they focused on a different aspect of clay mineral ingestion, the distribution of hazardous chemical elements into organs. Mascolo et al. (1999) documented this fact analyzing the urine of rats fed with different clay minerals and concluded that the chemical elements entered in blood dealing with possible hazardous effects on organs and/or the whole body.

Powell et al. (2007) reported that the human oral exposure to exogenous microparticles appeared to be almost exclusively additives to food, pharmaceuticals and toothpaste and that they were chiefly aluminosilicates and titanium dioxide. These particles are very resistant to chemical breakdown so, following ingestion and mucosal uptake, they survive both gastrointestinal digestion and cellular processing. Authors hypothesized that exogenous microparticles hijack the putative route for endogenous microparticle uptake but, due to their chemically-resistant nature, they cannot then

contribute to tolerance induction and may even, in susceptible individuals, establish abnormal immune responsiveness. In the present study IL-6 in serum has been not detected but a single parameter is not enough to conclude the absence of effects on the immune system.

It has been reported that hemolytic activity of silicate minerals decreases in the order smectites>silica>palygorskite ≈sepiolite>chrysotile>kaolinite (Oscarson et al., 1986; Carretero et al., 2006). Clay1 is based on Mt, a clay mineral of the smectites family, but in this case no hemolytic activity and no changes in the clinical biochemistry parameters of the animals after the experimental period has been detected.

5. Conclusion

The morphologic study performed on the tissues of Wistar rats exposed to 40 mg/kg/day Clay1 for 90 days did not evidence any remarkable effect. Similarly, the basic clinical biochemistry parameters, the GSH/GSSG ratio and the IL-6 leakage in serum did not show significant alterations.

Acknowledgements

The authors wish to thank the Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study, and the Clinical Biochemistry Unit of the University Hospital Virgen Macarena for the analysis performed on serum samples.

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Figure captions

Figure 1. Histopathological changes of Wistar rats exposed to 40 mg/kg/day Clay1 for 90 days. HE-stained sections of liver, kidney, lung, spleen, brain, testes, intestine and heart.

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Figure 3. Blood GSH/GSSG ratio in control Wistar rats and rats exposed to 40 mg/kg/day Clay1 for 90 days. Results are expressed as mean ± s.d.

Table 1

Click here to download Table: Table 1 Organ Weights Somatic.docx

	Control Group Mean ± SD	40 mg/kg/day Clay1 Mean ± SD
Liver (g)	13.12 ± 2.44	12.84 ± 1.67
Liver Somatic Index (%)	2.83 ± 0.40	2.53 ± 0.30
Kidneys (g)	3.02 ± 0.63	2.89 ± 0.26
Kidney Somatic Index (%)	0.65 ±0.11	0.60 ± 0.05
Lungs (g)	2.75 ± 0.78	2.72 ± 0.66
Lung Somatic Index (%)	0.60 ± 0.09	0.53 ± 0.12
Spleen (g)	1.02 ± 0.17	1.04 ± 0.13
Splenic Somatic Index (%)	0.22 ± 0.02	0.20 ± 0.02
Brain (g)	2.06 ± 0.18	2.14 ± 0.22
Brain Somatic Index (%)	0.45 ± 0.05	0.42 ± 0.05
Testes (g)	3.66 ± 0.75	3.77 ± 0.52
Testicle Somatic Index (%)	0.79 ± 0.11	0.75 ± 0.14
Intestine (g)	4.99 ± 1.022	5.68 ± 1.86
Intestine Somatic Index (%)	1.10 ± 0.37	1.00 ± 0.50
Heart (g)	1.82 ± 0.12	1.75 ± 0.19
Heart Somatic Index (%)	0.40 ± 0.05	0.34 ± 0.04

Table 1. Organs weight (g) and somatic index (%) of the different organs of control Wistar rats and rats exposed to 40 mg/kg/day Clay1 for 90 days. Results are expressed as mean ± sd.

Table 2

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Parameters	Control	40 mg/kg/day Clay1
	Mean ± SD	Mean ± SD
Glucose (mg/dL)	141.83 ± 61.95	149.75 ± 20.32
Urea (mg/dL)	58.00 ± 7.95	60.75 ± 7.50
Creatinine (mg/dL)	0.78 ± 0.08	0.78 ± 0.03
Total protein (g/dL)	6.17 ± 0.19	6.13 ± 0.25
Albumin (g/dL)	4.12 ± 0.22	4.10 ± 0.24
Cholesterol (mg/dL)	90.00 ± 15.61	83.50 ± 16.62
Aspartate Aminotransferase (AST) (U/L)	187.83 ± 67.29	189.75 ± 17.97
Alanine Aminotransferase (ALT) (U/L)	52.67 ± 16.28	49.50 ± 11.21
Alkaline Fosfatase (ALP) (U/L)	185.67 ± 54.64	193.50 ± 46.22
Sodium (mEq/L)	152.67 ± 1.21	151.00 ± 1.15
Potassium (mEq/L)	7.68 ± 1.12	7.40 ± 1.31

Table 2. Clinic biochemistry parameters of control Wistar rats and rats exposed to 40 mg/kg/day Clay1 for 90 days. Results are expressed as mean ± sd.

Figure 1

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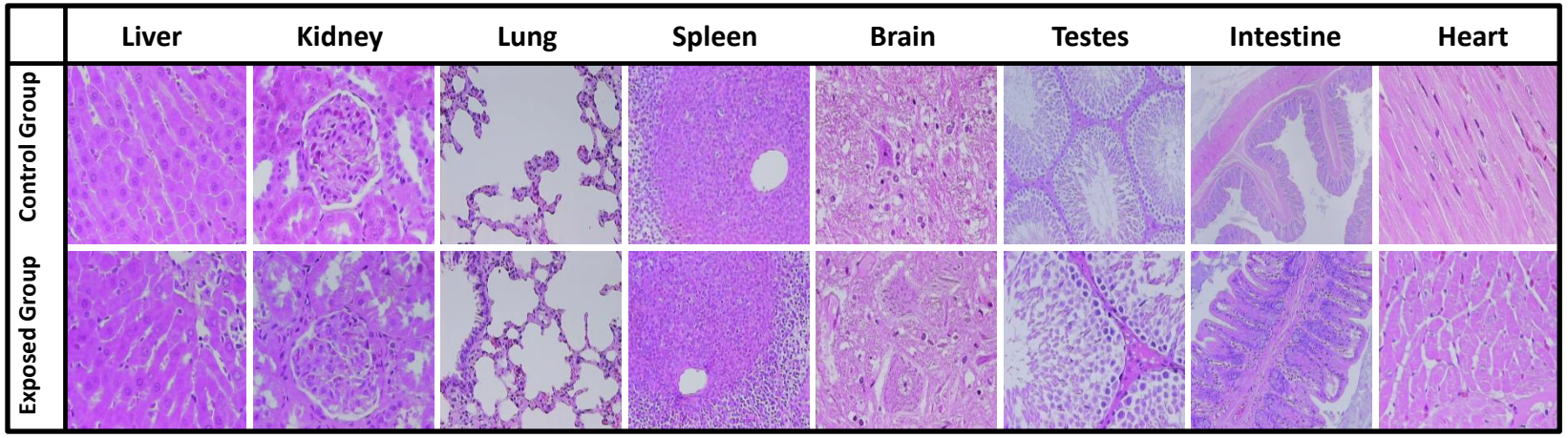

Figure 2

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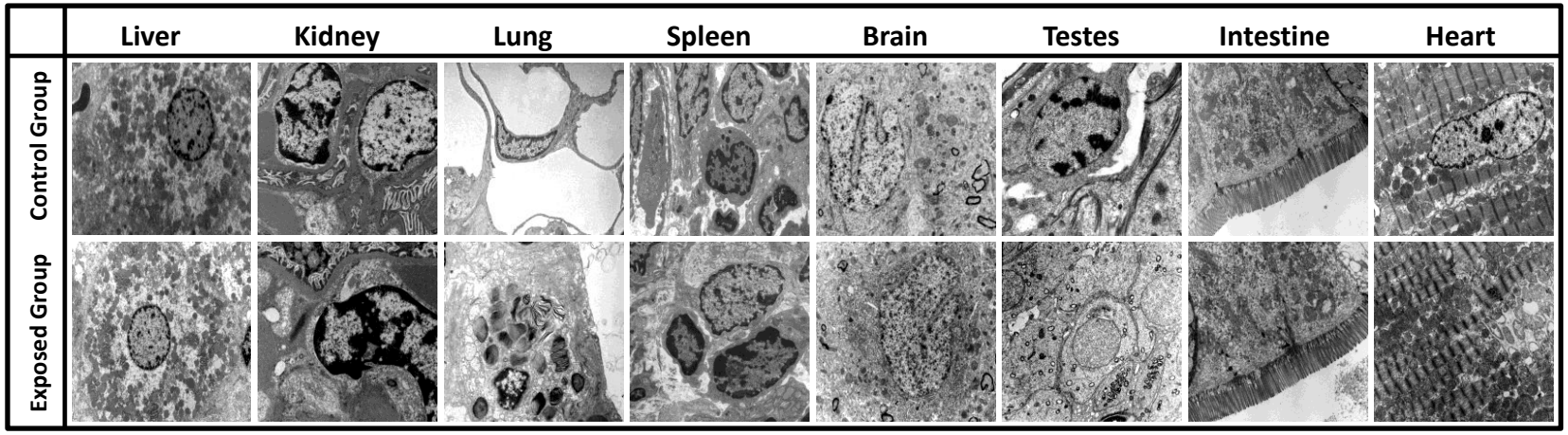
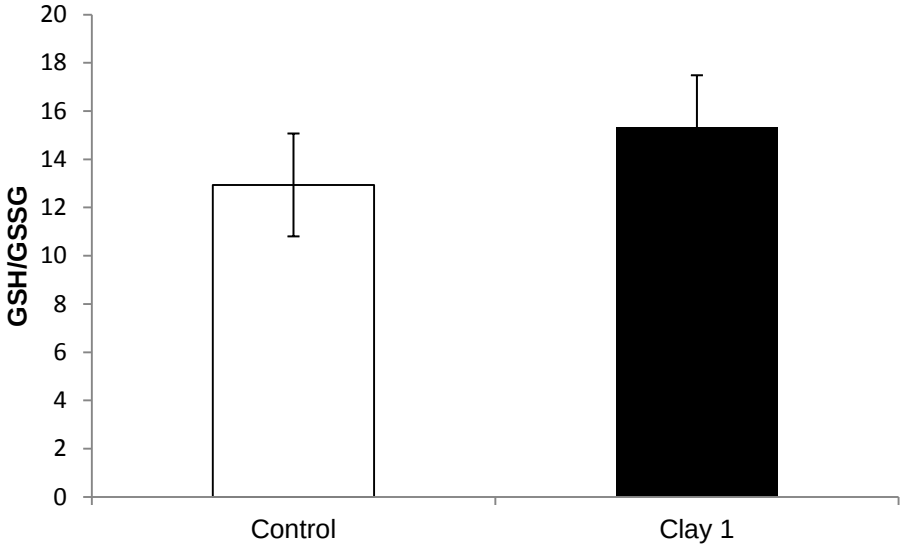


Figure 3

Click here to download Figure: Fig 3 GSH-GSSG FRI.pptx



CAPÍTULO 11/ CHAPTER 11

Sara Maisanaba, María Puerto, Daniel Gutiérrez-Praena, María Llana-Ruíz-Cabello, Silvia Pichardo, Alfonso Mate, María Jordá-Beneyto, Ana M. Cameán, Susana Aucejo, Ángeles Jos

IN VIVO EVALUATION OF ACTIVITIES AND EXPRESSION OF ANTIOXIDANT ENZYMES IN WISTAR RATS EXPOSED FOR 90 DAYS TO A MODIFIED CLAY

Journal of Toxicology and Environmental Health, Part A: Current Issues 77, 456-466, ***2014***

In vivo evaluation of activities and expression of antioxidant enzymes in Wistar rats exposed for 90 days to a modified clay

Journal:	*Journal of Toxicology and Environmental Health, Part A: Current Issues*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Maisanaba, Sara; Universidad de Sevilla, Área de Toxicología Puerto, María; Universidad de Sevilla, Área de Toxicología Gutiérrez-Praena, Daniel; Universidad de Sevilla, Área de Toxicología Llana-Ruíz-Cabello, María; Universidad de Sevilla, Área de Toxicología Pichardo, Silvia; Universidad de Sevilla, Área de Toxicología Mate, Alfonso Mate; Universidad de Sevilla, Jordá-Beneyto, María; ITENE, ; ITENE, Cameán, Ana; Universidad de Sevilla, Área de Toxicología Aucejo, Susana; ITENE, Jos, Ángeles; Universidad de Sevilla, Área de Toxicología
Keywords:	clay, food packaging; , organomodified clay, oxidative stress, rat

In vivo evaluation of activities and expression of antioxidant enzymes in Wistar rats exposed for 90 days to a modified clay

Sara Maisanaba[1], María Puerto[1], Daniel Gutiérrez-Praena[1], María Llana-Ruíz-Cabello[1], Silvia Pichardo[1*], Alfonso Mate[2], María Jordá-Beneyto[3], Ana M. Cameán[1], Susana Aucejo[3], Ángeles Jos[1]

[1]Area de Toxicología, Facultad de Farmacia, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

[2]Deparmento de Fisiología, Facultad de Farmacia, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

[3]Area of Packaging Materials and Systems, ITENE, C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

*Corresponding author:

Silvia Pichardo

Area de Toxicología, Facultad de Farmacia, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: spichardo@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Clays have uses in a wide range of applications. Despite their increasing use, the toxicity assessment of these new materials is still scarce. In the present work, the oxidative stress induced by Clay 1, a novel clay, has been evaluated in rats after 90 days of oral exposure. The activities of the antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were studied. In addition, the genetic expression of SOD and CAT and the relative protein abundance of CAT were also evaluated. Our results showed that most of the biomarkers assayed stayed unaltered. Only CAT activity, as well as its genetic and protein expressions, appeared enhanced in the kidney. Therefore, further studies are needed to clarify the consequences of this finding to ensure the safety of this clay.

Keywords: clay; food packaging; organomodified clay; oxidative stress; rat

Introduction

Clays and polymer-layered nanocomposites have uses in a wide range of applications (Lordan et al. 2010). Organically modified clays, or organoclays are currently employed in the manufacture of inks, paints, greases and cosmetics, in water treatment applications (Patel et al. 2006), controlled release of therapeutic agents (Park et al. 2008), or in food packaging products, among others. Polymer composites incorporating clay particles were among the first nanocomposites to emerge on the market as improved materials for food packaging (Chaudhry et al. 2008). The surface modification of the clay minerals is the key to achieve new applications of these materials (Betega de Paiva et al. 2008). The result is the creation of polymer layered nanocomposites, one of the most currently developed areas in nanotechnology. Smectite clays, and particularly montmorillonite (MMT), are one of the best choices for designing new polymer nanocomposites. Some of their advantages include low price worldwide spread and easy intercalation chemistry, which allows them to be chemically modified in order to improve the compatibility with some different polymeric matrices (Bitinis et al. 2011). Moreover, their high surface area (700-800 m^2/g) and large aspect ratio (50-1000) make them effective reinforcement fillers (Majeed et al. 2013).

When clays are dispersed into the polymer, they acquire a natural layered structure. These layers have sub-micrometer dimensions, except for their thickness, which is only about 1 nm (Hatzigrigoriou and Papaspyrides 2011). The presence of clays in the polymer matrix generates a material with improved barrier properties due to the disposition of the clays, giving a tortuous path around the polymer for gases and

water vapor (Nielsen 1967). These properties are partially due to the dispersion level (or major exfoliation) of clays into the polymer, which is different for each one and depends on the cationic exchange capacity (CEC) of the clay (Dean et al. 2007). In the case of MMT, modifications by exchanging inorganic cations with organic ammonium ions are justified (Baek et al. 2012). These modifications lead to a more regular organization of the layers in the structure, thus enhancing the compatibility of the clay with the organic polymers, and decreasing the water uptake by the nanocomposite (Osman et al. 2003). As a result, these improvements lead to an increase in the shelf life of the products.

Although the presence of organoclays in polymers is growing for various applications, little is known about the potential physiological effects these clays can induce if they enter in the human organism. That is, despite the large applications of organoclays, toxicity studies are still scarce. In this sense, the oral pathway is the most likely route of intake for clays incorporated in food packaging. Currently, the European Food Security Agency (EFSA) requires a complete study about the toxic effects generated by nanostructures formed by clays incorporated in polymers and their migration from the packaging to the food and into the organism afterwards (EFSA 2011), in order to perform their risk assessment.

The toxicological analysis of clays can be performed both by *in vitro* and *in vivo* methods. *In vitro* methods are required by the European Authorities for ethical reasons, being the aim to perform a toxicity screening, evaluating potential cytotoxic effects as well as the identification of toxic mechanisms (EFSA 2011). Limited

information about toxicological effects caused by clays can be found in the literature, although some authors have studied their toxic effects in different cell lines. In this sense, Sharma et al. (2010) evaluated the cytotoxicity and genotoxicity of non-modified and modified MMT (Cloisite®Na^+ and Cloisite®30B, respectively) in the human intestinal Caco-2 cell line, concluding that the modifier of Cloisite®30B was responsible of the observed genotoxic effects. Moreover, Baek et al. (2012) also evaluated *in vitro* the toxic effects of MMT in human normal intestinal cells (INT-407), which experienced an inhibition of proliferation in a concentration- and time-dependent manner. Furthermore, Lordan et al. (2011) observed that Cloisite®Na^+ and Cloisite®93A reduced the viability of human hepatoma HepG2 cells. In addition, our research group has obtained different toxic profiles of several modified and unmodified clays in Caco-2 and Hep-G2 cells (Houtman et al. 2013; Maisanaba et al. 2013a, 2013b). This suggests that a specific toxicity evaluation is necessary for each clay, and also that modifiers may be involved in the toxicity of polymer clays.

Most of the few assays performed so far *in vivo* reference to studies with a short exposure time and a high dose of clays (Mascolo et al. 2004; Wang et al. 2005), which is not representative of the chronic exposure that could occur in a daily consumer. Due to the scarce number of studies, the toxic mechanism for these clays is still unknown. One of the principal mechanisms that could initiate adverse biological responses, leading to toxicological effects of manufactured nanoparticles, is the ability to cause oxidative stress (Lordan et al. 2010). Therefore, the purpose of this study was to assess a possible implication of the novel organoclay, Clay 1, in the induction of oxidative stress in Wistar rats exposed to 40 mg/kg/day of Clay 1 for 90 days. An

evaluation of the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), was performed. In addition, the genetic expression of SOD and CAT, and the relative protein abundance of CAT, were also evaluated.

Materials and Methods

Supplies and Chemicals

General chemicals were provided by Sigma Aldrich (Madrid, Spain) and VWR International Eurolab (Spain). The organomodified clay encoded as Clay 1 was obtained by cation exchange reaction from Cloisite ®Na^+ (Southern Clay Products, INC.) adding the quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) in 6 fold the cation exchange capacity (CEC) of pristine clay, following the method described by Jordá-Beneyto et al. (2013). Briefly, the modified clay was characterized by Fourier Transform InfraRed (FTIR) spectroscopy, thermogravimetric analysis (TGA) and X-ray diffraction (XRD). Regarding FTIR analysis, the presence of typical absorption bands attributed to Si-O-Si and Al-O-Si stretching vibrations were observed at 910, 972 and 1118 cm^{-1}. Furthermore, hydroxyl groups bonded to Al and/or Mg (3620 cm^{-1}), the methylene symmetric and antisymmetric stretching bands (3020-2800 cm^{-1}) and the characteristic bending vibration of the alkylammonium cation (1487 cm^{-1}) were also shown in the FTIR spectrum. The amount of organic cation in the modified Cloisite ®Na^+ was determined by TGA. Thermograms revealed a loss weight (%) of 24% for Clay 1 in the temperature range of 200-500°C, ascribed to hexadecylammonium thermal decomposition. Moreover, XRD patterns of Clay 1 showed an expanded interlayer d(001)-spacing of 18.00 Å (peak position at 2θº=4.93 in X-ray diffractogram) compared

to that displayed by the pristine Cloisite ®Na^+, d(001)-spacing of 12.1 Å (2θ0=7.31). These data and additional characterization data of Clay 1 are available in Jordá-Beneyto et al. (2013).

Experimental animals and tissue sampling

A repeated dose 90-day oral toxicity study was performed in rats. Briefly, twenty male Wistar rats provided by Janvier S.A.S (France) with a mean weight of 240.2 ± 2.04 g were used. Rats were fed during the acclimation week with a standard laboratory diet (Harlan 2014, Harlan Laboratories, Barcelona, Spain) and water, both *ad libitum*, in a controlled temperature room (23 ± 1ºC) with 12 h dark/light cycle, and free from any source of chemical contamination. After this period, animals were randomly divided in two groups, a control group (n=10) and an exposed group (n=10). The control group was fed with the standard diet, while the exposed group was orally administered with 40 mg/kg/day of Clay 1 in the diet. This dose was selected to reproduce 2-fold the highest dose of the worst case scenario of human exposure (that is, assuming that all the clay incorporated into the packaging polymer was transferred into the food). During the treatment period clinical signs, body weight, and food and water consumption were recorded weekly.

All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes, and all the procedures were previously accepted by the Ethic Committee of the University of Seville.

At the end of the experimental period, rats were fasted for 18 h before sacrifice. Liver and kidneys were excised, rinsed with cold saline solution and weighted. Briefly, tissues were homogenized using 50mM phosphate monobasic, 1mM tetrasodium dihydrate salt and bovine serum albumin (BSA) buffer (pH 7,5) at 0-4 ºC with a polytron homogenizer (Heidolph RZR2102 Control, Sigma, Madrid, Spain). The homogenates were centrifuged 20 min at 4000 rpm at 4°C. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for enzyme measurements.

Protein estimation

Protein contents in the samples were estimated by the method of Bradford (1976) using bovine γ-globulin as standard. Briefly, 5 µL of the diluted samples were mixed with 245 µL Coomassie brilliant blue dye (Biorad Laboratories, Hercules, USA) and the absorbance was read at 595 nm in the microplate reader (Tecan Infinite M200, Austria).

Lipid peroxidation

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman 1990). Malondialdehyde (MDA) was formed as a lipid peroxidation end-product that reacts with TBA reagent under acidic conditions to generate a pink coloured product. Briefly, 0.5 mL of the homogenized tissue, obtained previously, was treated with 25 µl of butylhydroxytoluene 1% (v/v) in acetic acid and mixed with 0.2 mL of sodium lauryl sulphate (8%), 1 mL of acetic acid (20% v/v) and 1 mL of 0.8% thiobarbituric acid. This mixture was then heated at 95°C for 30 min. The resulting chromogen was extracted with 3 mL of 2-butanol and, after centrifugation

(1500 x g for 10 min), the absorbance of the organic phase was determined at 532 nm. 1,1,3,3- Tetraethoxypropan (TEP) was used as a standard. Values were expressed as nmol of thiobarbituric acid reactive substances (TBARs)/ g tissue.

Antioxidant enzymes activities

Catalase (Enzyme Commission [EC] 1.11.1.6) activity was assayed in liver and renal homogenates following the method described by Beers and Sizer (1995), measuring the initial rate of H_2O_2 (10 mM) decomposition at 240 nm. Total superoxide dismutase (EC 1.15.1.1) activity was determined based on the method described by McCord and Fridovich (1969). Glutathione peroxidase (EC 1.11.1.9) activity was assayed by following the rate of reduced nicotinamide adenine dinucleotide phosphate oxidation at 340 nm by the coupled reaction with glutathione reductase. The specific activity was determined using the extinction coefficient of 6.22/mM/cm (Lawrence and Burk, 1976). Glutathione S-transferase activity (EC 2.5.1.18) was measured according to the method described by Habig et al. (1974), using 1-chloro-2,4- dinitrobenzene as a substrate. All enzymatic activities are expressed in nKat/mg protein.

Genetic expression analysis

RNA preparation and reverse transcription

Total RNA was extracted and purified using the RNAeasy Mini Kit™ (Qiagen, Madrid, Spain) according to the manufacturer's instructions. The RNA integrity was assessed by agarose gel electrophoresis. RNA quality was assessed from the 260/280 nm absorbance ratio using a NanoDrop 2000 (Thermo Scientific, USA). The RNA was then stored at 80ºC. The RNA transcription into cDNA was carried out with a High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions. The cDNA was stored at -20ºC until further use.

Reverse transcription and real-time polymerase chain reaction (RT-PCR) for CAT and SOD

A semi-quantitative RT-PCR protocol was developed to measure the mRNA levels of SOD and CAT in liver and kidney, using GAPDH as protein control. The cDNA obtained was diluted in milliQ sterile water and used for amplification by real-time PCR. The forward and reverse primers used in this study are shown in Table 1. All mRNA sequences for SOD, CAT and GAPDH were obtained from GenBank. PCR primers were obtained from Sigma-Aldrich (Madrid, Spain). Each specific gene product was amplified by RT-PCR using a LightCycler®480 System (Roche, Germany) according to the following parameters: 50ºC for 2 min, 95ºC for 10 min, 95ºC for 15 s , 60ºC for 1 min (40 cycles) and 72ºC for 10 min (40 cycles), and 4ºC until the pickup of the samples. Amplification data were collected and analyzed with the software supplied by the manufacturer. The quantitative fold changes in mRNA expression were determined relative to the GAPDH mRNA levels in each corresponding group and calculated using the $2^{-\Delta\Delta CT}$method (Gutiérrez-Praena et al. 2013).

Western blot analysis for CAT

Liver and kidney tissues were prepared for the measurement of CAT abundance by a Western blot analysis. Liver and kidney homogenized samples were mixed (1:1) with sample buffer containing β-mercaptoethanol (5%). Proteins were electrotransferred onto nitrocellulose membranes and inmunoblotted as described by Gutiérrez-Praena

et al. (2013) using monoclonal anti-CAT antibodies (Sigma-Aldrich, Madrid, Spain), diluted at 1/10000. The anti-CAT was detected by the enhanced chemiluminiscence (ECL) method according to the supplier´s protocol (Sigma, Madrid, Spain) and using a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, USA) as secondary antibody (1/10000).

Statistics

Data are expressed as mean ± standard deviation of ten animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

Results

No rat died during the experimental period and no remarkable clinical or behavioral signs were observed. Moreover, body weight gain, and food and water consumption was unaltered by exposure to Clay 1.

Lipid peroxidation

MDA levels remained unaltered in rats fed with Clay 1 in comparison to the control group in liver and kidney (Fig. 1).

Antioxidant enzymes activities

No significant changes in CAT activity were recorded in the liver of rats exposed to Clay 1 (Fig. 2A). However, in the kidney, CAT activity underwent a significant enhancement in comparison to the control group (Fig. 2B). Concerning the activities of

the other antioxidant enzymes (SOD, GPx, and GST), no statistical differences were observed in any of the studied organs (Figs. 2C, 2D, 3).

RT-PCR for CAT and SOD

In liver of rats orally exposed to Clay 1, no significant alterations were observed in the relative gene expression of CAT (Fig. 4A). Meanwhile, a 4-fold enhancement of the transcription of this enzyme was observed in kidney (Fig. 4B). In the case of SOD, the relative gene expression was unaltered in the liver (Fig. 4C). However, although the gene expression of SOD was increased in kidney, it was not significant in comparison to the control group (Fig. 4D).

Western blot analysis for CAT

Considering that only CAT showed alterations in both, the activity and the relative gene expression, the analyses of its protein expression was also performed. Similarly, no effect was recorded in the case of liver in the Western blot assay (Fig. 5A); on the other hand, there was a significant increase in the protein expression of CAT in the kidney of rats fed with Clay 1 (Fig. 5B).

Discussion

The expanding use of MMT in different applications including food packaging makes it unavoidable an evaluation of its potential toxicity. The present work aims to assess the safety of an organomodified MMT, namely Clay 1, which has been manufactured by the Packaging, Transport, & Logistics Research Institute (ITENE, Valencia, Spain). It has been developed to reinforce food packaging materials, thus it is

potentially suitable for food contact materials (Jordá-Beneyto et al. 2013). Previous studies performed in our laboratory have showed that Clay 1 had low cytotoxic effects and no genotoxicity at the concentrations assayed up to 8 µg/mL on the human permanent cell lines Caco-2 and HepG2 (Houtman et al. 2013). Higher concentrations could not be assessed *in vitro*, due to its low solubility that interfered with the measurement system (i.e., spectrophotometry). In order to assess its safety, and considering the lack of information for this clay, an oral sub-chronic toxicity assay has now been performed.

In our study, no rat died during the experimental period, nor remarkable clinical signs were observed. Moreover, a histopathological study of the main organs of these animals did not show toxic injure (Maisanaba et al. 2013c). Similarly, mice orally exposed up to a single dose of 1000 mg/kg of MMT did not show any remarkable abnormal behavior, symptons or body weight loss during 14 days post-administration (Baek et al. 2012). Also, Mascolo et al. (2004) reported no macro-toxic effects during a 6-day trial on male Wistar rats orally exposed to bentonite and black shale. In fact, the safety of different clays has been already stated. Wang et al. (2005) reported that a Ca-MMT was safe on a group of volunteers during a short term experiment (2 weeks). Moreover, clays have been reported to have positive effects in several *in vivo* experiments. Thus, Cu^{2+}-exchanged montmorillonite exhibited antibacterial activity *in vivo* and protected the intestinal mucosa from the invasion of pathogenic bacterium and toxins, resulting in a positive effect on the growth performance (Hu et al. 2007). Additionally, the external utilization of bentonite for wound healing was found to be safe and feasible on rats (Emami-Razavi et al. 2006). However, human intestinal cells

exposed to a MMT for 10 days underwent significant damage; while no remarkable toxic effects were found in mice receiving up to 1000 mg/kg of this MMT (Baek et al. 2012). Therefore, *in vivo* experiments are needed in order to verify the toxic effects observed in the *in vitro* assays. Moreover, considering the differences in the chemical structure for each organomodified clay, the toxic profile could also be different, thus making necessary a toxic assessment on an individual basis.

Concerning the toxic mechanism of clays, some *in vitro* experiments have already suggested the implication of the oxidative stress in the pathogenicity of MMT, since it has been pointed out as one of the principal toxic mechanisms of manufactured nanoclays (Lordan et al. 2010). Oxidative stress occurs in a cell or organism when there is an imbalance between the generation and removal of reactive oxygen species (ROS), resulting in DNA, lipid and protein damage. To keep destructive free radical levels low, the cell has enzymatic antioxidant defences (e.g., SOD, CAT, GPx, GST) and non-enzymatic defences, such as GSH (Puerto et al. 2010). Lordan et al. (2010) reported that Cloisite®Na^+ induced intracellular ROS formation, which coincided with increased cell membrane damage. However, on the intestinal cell line Caco-2, neither Cloisite®Na^+ nor the organo-modified MMT Cloisite®30B produced ROS (Sharma et al. 2010). Moreover, our research group found no alterations on the ROS values in any of the cell lines (HepG-2 and Caco-2) exposed to Clay 2, a similar clay as the one used in this study that also contains as modifier an acetylcholine chloride group, although a decrease in the GSH levels in HepG2 cells was observed (Houtman et al. 2013). Furthermore, Baek et al. (2012) evaluated the ROS production in INT-407 cells exposed to MMT, obtaining significant levels of ROS at the highest concentration

(1000 µg/ mL) at the three timepoints assayed (24, 48 and 72h). Therefore, the modifiers used in the organoclays contributed to the toxic effects (Houtman et al. 2013; Maisanaba et al. 2013a, 2013b). Malondialdehyde (MDA) is often used as a marker of the lipid peroxidation (Esterbauer and Cheeseman 1990) and is therefore an indicator of oxidative damage in cell membranes. In the present study, no alterations in the MDA levels nor in the antioxidant enzymes (SOD, GPx and GST) were recorded. Similarly, Shi et al. (2006) reported no changes in the SOD, GPx, and MDA levels in liver and serum of broiler chickens orally exposed to a modified MMT nanocomposite. On the contrary, the addition of MMT to a diet containing aflatoxin restored the antioxidant capacity of the chicks. Likewise, MDA content decreased in carps exposed to a Cd-contaminated basal diet supplemented with MMT in comparison with the Cd-contaminated group (Kim et al. 2011). In the present work, although no significant changes in the activities of SOD, GPx, and GST were recorded in liver and kidney, CAT activity underwent a significant enhancement in kidney.

The transcriptional regulation of CAT induced by Clay 1 in kidney agrees with the protein abundance observed in this organ. Besides, the quantity of the enzyme detected by Western blot analysis coincides with the activity of this enzyme. Therefore, our results show an adaptive response of rats exposed to Clay 1, which increases antioxidant enzymatic activity of CAT to overwhelm the oxidative insult. This finding is in contrast to the lack of histopathological lesions observed in liver and kidney, which may indicate the need for a higher change in the biomarkers assayed for the onset of histological changes. In fact, rats gavaged with sediment consisting of 15% MMT clay and hexachlorbenzene showed normal histochemical examinations while biochemical analysis did evidence damage (Lee et al. 2005).

Finally, it would be interesting to clarify the organospecificity of clays. In this sense, MMT (Cloisite®Na^+) has recently been reported to be absorbed into the body within 2 h, without significant accumulation in any specific organ (Baek et al. 2012). However, Mascolo et al. (2004) showed that the distribution of trace elements from clays in male Wistar rats fed with bentonite and black shale was not homogeneous throughout the body, finding the highest amounts of them in kidney followed by liver. This could explain, in part, the findings observed in our study, where CAT activity was altered only in kidney.

Conclusion

Considering the results obtained, we cannot conclude that Clay 1 induces oxidative stress in rats orally exposed to it, since most of the biomarkers assayed stayed unaltered. However, taking into account that CAT activity, as well as its genetic and protein expression were enhanced, it is undeniable that Clay 1 is able to induce some alterations. Therefore, further studies are needed to clarify the consequences of our findings in order to ensure the safety of this clay.

Acknowledgments

The authors wish to thank Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study.

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Table captions

Table 1. Nucleotides sequences (5'-3') of PCR primers of GAPDH, SOD, and CAT.

Figure captions

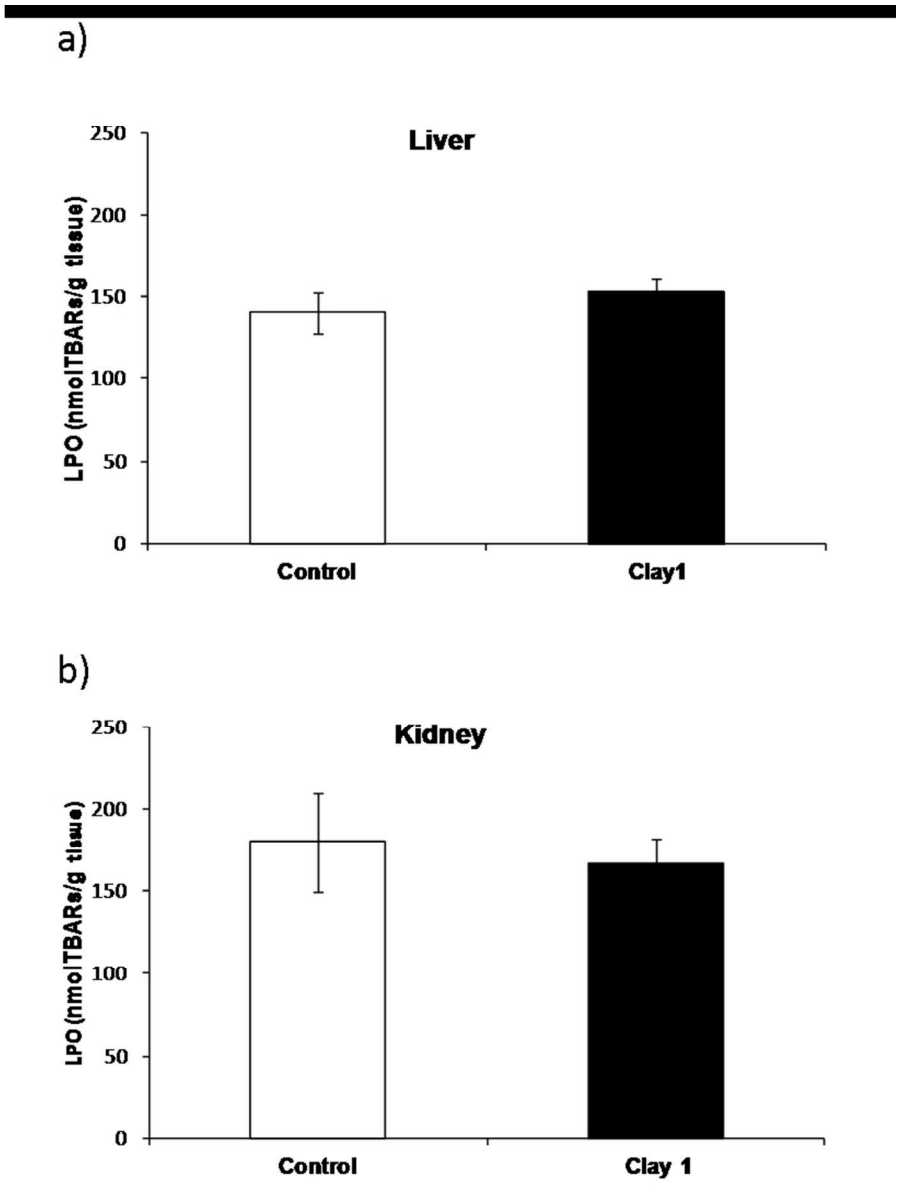
Figure 1. Effect of Clay 1 on lipid peroxidation on liver (a) and kidney (b) of rat. The values are expressed as mean±S.D. (n=10). LPO value is expressed as nmol TBARs/g tissue.

Figure 2. Catalase (CAT) and superoxide dismutase (SOD) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to Clay 1. The values are expressed as mean±S.D. (n=10). The significance levels observed are **$p < 0.01$ in comparison to control group values.

Figure 3. Glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to Clay 1. The values are expressed as mean±S.D. (n=10).

Figure 4. Relative gene expression of catalase (CAT) and superoxide dismutase (SOD) in liver (a,c) and kidney (b,d) of rat exposed to Clay 1. The values are expressed as mean±S.D. (n=10). The significance levels observed are ***$p < 0.001$ in comparison to control group values.

Figure 5. Relative abundance of catalase (CAT) protein in liver (a) and kidney (b) of rat exposed to Clay 1. The values are expressed as mean±S.D. (n=10). The significance levels observed are *$p < 0.05$ in comparison to control group values.



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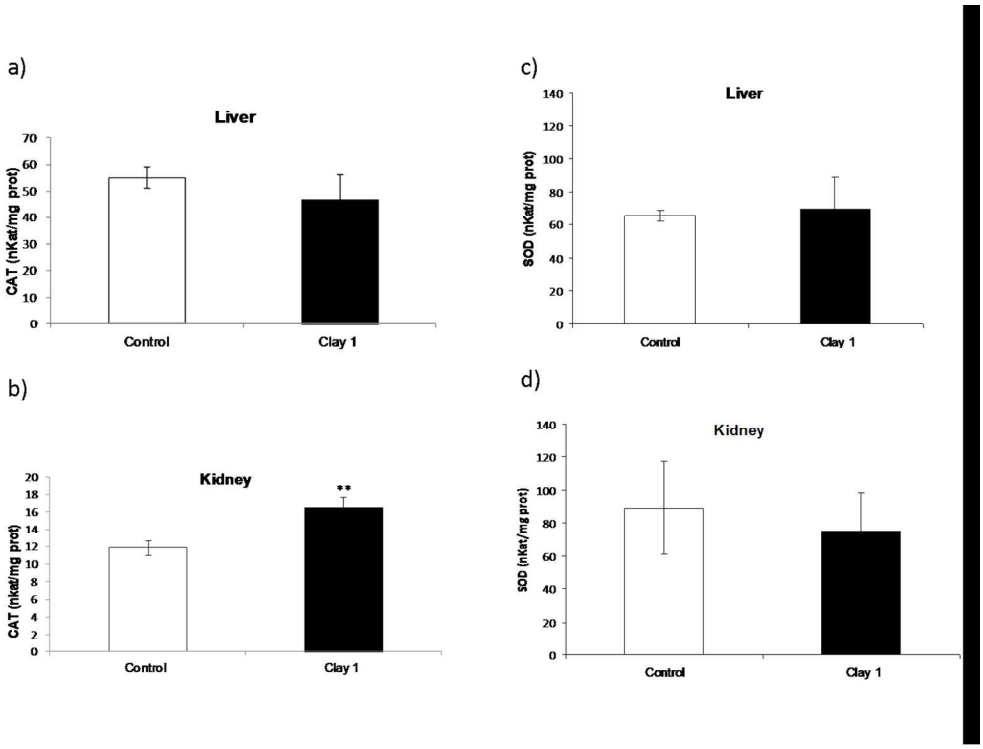

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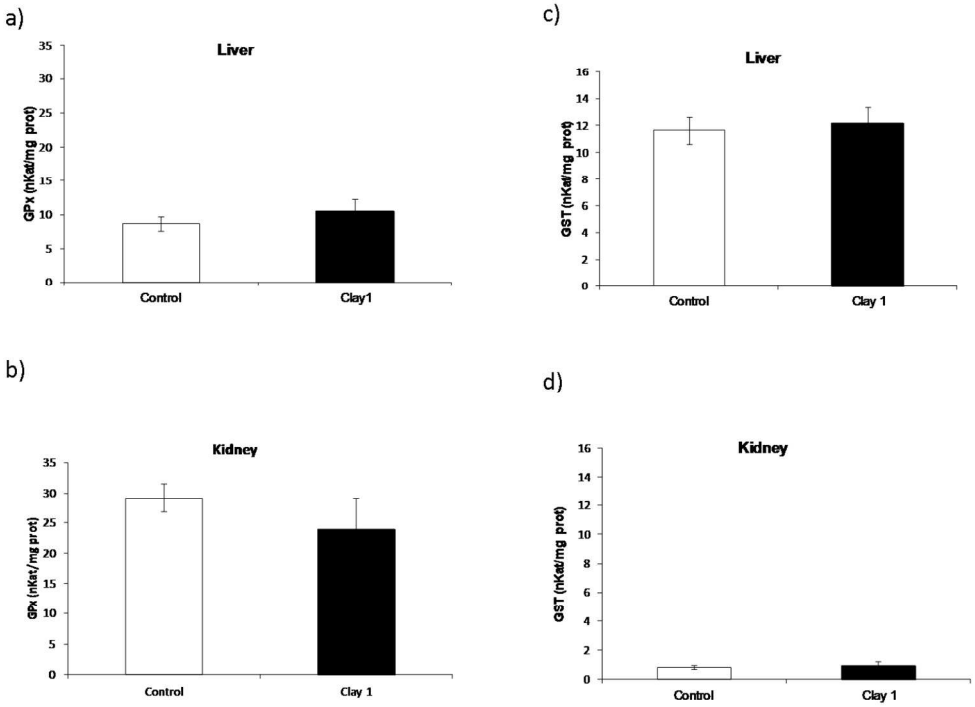

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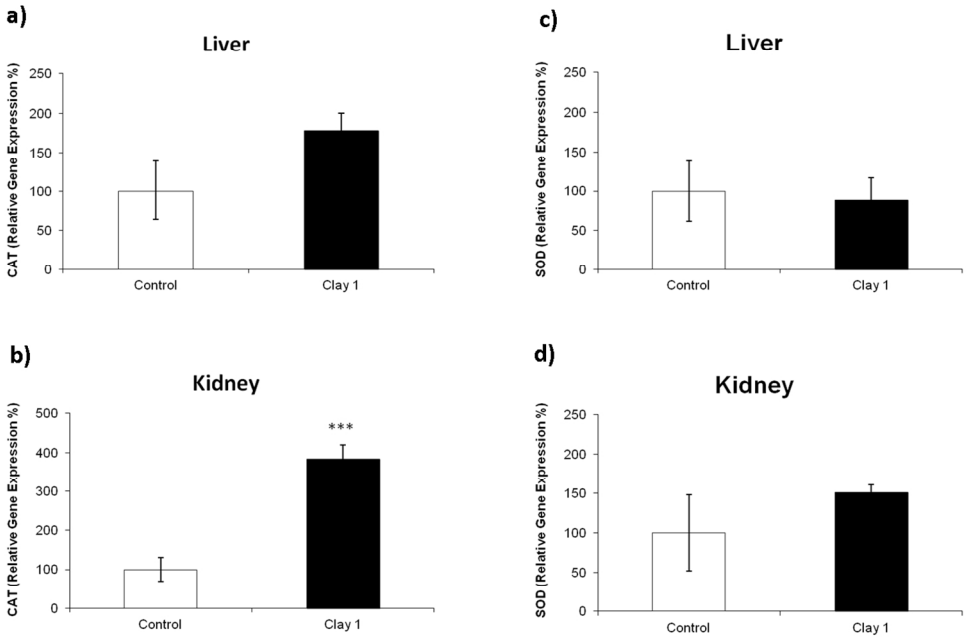

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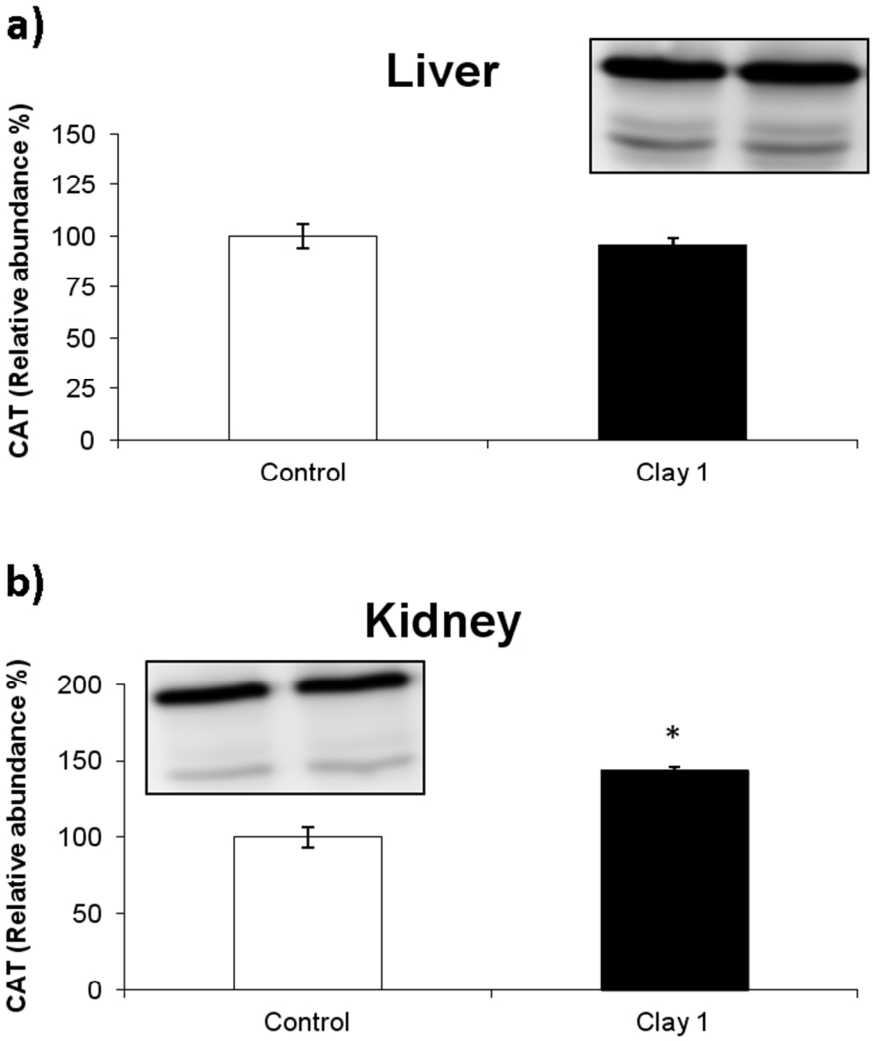

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Table 1. Nucleotides sequences (5'-3') of PCR primers of GAPDH, SOD, and CAT

Gene*	Forward primer	Reverse primer
GAPDH	GCCAAAAGGGTCATCATCTCCGC	GGATGACCTTGCCCACAGCCTTG
SOD	CGTCATTCACTTCGAGCAGAAGG	GTCTGAGACTCAGACCACATA
CAT	CCTTTATCCATAGCCAGAAG	AGCGTGAGTCTGCGCTTCAG

* GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SOD: Superoxide dismutase; CAT: Catalase

CAPÍTULO 12/ CHAPTER 12

Sara Maisanaba, Daniel Gutiérrez-Praena, María Puerto, María Llana-Ruíz-Cabello, Silvia Pichardo, Rosario Moyano, Alfonso Blanco, María Jordá-Beneyto, Ángeles Jos

IN VIVO TOXICITY EVALUATION OF THE MIGRATION EXTRACT OF AN ORGANOMODIFIED CLAY-POLY(LACTIC) ACID NANOCOMPOSITE

Journal of Toxicology and Environmental Health, Part A: Current Issues 77, 731-746, ***2014***

In vivo toxicity evaluation of the migration extract of an organomodified clay- poly(lactic) acid nanocomposite

Journal:	*Journal of Toxicology and Environmental Health, Part A: Current Issues*
Manuscript ID:	UTEH-2014-0027
Manuscript Type:	Original Article
Date Submitted by the Author:	28-Jan-2014
Complete List of Authors:	Maisanaba, Sara; University of Sevilla, Gutiérrez-Praena, Daniel; University of Sevilla, Area of Toxicology Puerto, María; University of Sevilla, Area of Toxicology Llana-Ruíz-Cabello, María; Universidad de Sevilla, Área de Toxicología Pichardo, Silvia; University of Sevilla, Area of Toxicology Moyano, Rosario; University of Cordoba, Blanco, Alfonso; University of Cordoba, Jordá-Beneyto, María; Technological Institute of Packaging, Transport and Logistic, Jos, Ángeles; University of Sevilla,
Keywords:	Clay, Nanocomposite, Montmorillonite, Toxicity, Migration extract

PLA-Clay1 extract *in vivo* toxicity evaluation

In vivo toxicity evaluation of the migration extract of an organomodified clay-poly(lactic) acid nanocomposite

Sara Maisanaba[1], Daniel Gutiérrez-Praena[1*], María Puerto[1], María Llana Ruiz-Cabello[1], Silvia Pichardo[1], Rosario Moyano[2], Alfonso Blanco[3], María Jordá-Beneyto[4], Ángeles Jos[1]

[1] Area of Toxicology, Faculty of Pharmacy, University of Sevilla, C/ Profesor García González 2, 41012 Seville, Spain.

[2] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, Faculty of Veterinary, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[3] Department of Anatomy and Comparative Pathology and Anatomy, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, 14071 Córdoba, Spain.

[4] Area of Packaging Materials and Systems, Technological Institute of Packaging, Transport and Logistic (ITENE), C/ Albert Einstein 1, 46980 Paterna (Valencia), Spain.

* Corresponding author:

Daniel Gutiérrez-Praena. Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González 2, 41012 Seville, Spain. Tel: +34 954 556762; Fax: +34 954 556422

E-mail: dgpraena@us.es

Abstract

Food packaging industry is in a continuous development in order to obtain more secure and stable food and beverages. The incorporation of inorganic and organic materials to plastic polymers leads to polymer-composites. Among inorganic compounds, clays such as montmorillonite (MTT) and its derivatives are of great interest due to their advantageous properties. The Technological Institute of Packaging, Transport and Logistic (ITENE) has developed a novel nanocomposite based on a poly (lactic) acid (PLA) polymer using a MMT-derivate, named Clay1, as filler, to be used in the beverage industry. The improvement of the technological properties of this new material has been demonstrated but safety issues are also of concern. In the present work, a histopathological study by optical and electron microscopy of organs from Wistar rats exposed during 90 days to a migration extract of PLA-Clay 1 nanocomposite, was carried out. Moreover, different clinical biochemistry, inflammation and oxidative stress biomarkers were evaluated. Results showed no evidence of damage, indicating that this nanocomposite has a good profile to be used in the food packaging industry, although further research is still needed.

Keywords: clay, nanocomposite, montmorillonite, toxicity, migration extract

Introduction

Food market demands technology advances, which are essential to keep the market leadership in the food processing industry, in order to produce fresh, convenient and flavourful food products (Alfadul et al., 2010). Nanotechnology can possibly improve production processes, providing products with better characteristics and new functionalities in food and bio-processing industries (Roco, 2002). One of the potential application and possible concern over the use of nanomaterials and nanotechnology in food industry is the development of polymer nanocomposites, which are very useful in the food packaging industry. Hence, nanomaterials are incorporated in to the packaging to improve physical performance, durability, barrier properties, biodegradation, etc. (Bradley et al., 2011; Neethirajan et al., 2011).

Among different kinds of materials used in food packaging industry, the use of composites must be emphasized. Composites typically consist of a polymer matrix or continuous phase and a discontinuous phase or filler (Matthews et al., 1994). In this way, nanocomposites are materials in which the filler has at least one dimension smaller than 100 nm (Arora et al., 2010). The mixture of polymers with inorganic and organic additives results in polymer-composites, which present certain geometries. In order to create novel polymer composites, various inorganic nanoparticles have been recognized as possible additives to enhance polymers performance (Sorrentino et al., 2007). Among these, organoclays are the most dominant commercial materials to prepare this kind of nanomaterials (Markarian, 2005). One of the most used clays to form these organoclays has been montmorillonite (MMT), a natural phyllosilicate. This compound exhibits excellent properties, such as high cation exchange, swelling behaviour, good adsorption properties and large surface area (Betega de Paiva et al., 2008). Once the clays have

been incorporated into the polymer they become into nanoclays. These are defined as natural layered structures, where the layers or platelets have sub-micrometer dimensions, except for their thickness, which is only about 1 nm (Hatzigrigoriou et al., 2011). The main advantages of nanoclays are flame retardant synergy, improved balance of stiffness and toughness, and barrier properties (Markarian, 2005). The aforementioned properties could be explained through the most widely known theories to improved barrier properties of polymer-clay nanocomposites, described by Nielsen (1967). This theory focuses on a tortuous path around the clay plates, forcing the permeant gases to travel a longer path to diffuse through the film.

The improvements and benefits that polymer-clay nanocomposites could exhibit are a reality nowadays. However, the potential toxicological effects and impacts in the fields of human health and food safety are still unknown in many ways, being necessary to carry out studies in order to clarify these effects (Ellenbecker et al., 2011; Bouwmeester et al., 2009).

Engineered nanomaterials, nanoclays in this particular case, present specific physicochemical properties, whose should be correlated with a unique and specific toxicokinetics profile in each case. Moreover, these physicochemical and toxicological properties cannot be inferred from their bulk counterparts. But, on the other hand, these nanoclays will only reach the consumers if their migration from the package to the food is evidenced. According with the current legislation, all the aspects related to plastic materials and articles intended to come in contact with food and foodstuffs, as well as the simulants used in the migration assays are embraced in both the EU Commission

Regulation N° 10/2011 and the Council Directive 82/711/EEC. The final aim of these regulations is to ensure the safety of the final material or article.

The Technological Institute of Packaging, Transport and Logistic (ITENE) is developing modified-clays based on MMT to be incorporated into polymers. Thus, a novel nanocomposite with .poly(lactic) acid (PLA) and a modified organoclay (Clay1) has been created to be used in the beverage industry. The improvement of the technological aspects of this new material have been evidenced (Jordá-Beneyto et al., 2014) but there are no data yet on safety issues. Moreover, few studies are present in the literature about migration from nanocomposites used as packaging of food or beverages, and the toxicological effects of the migrants.

Taking into account the previous background, this study provides an *in vivo* toxicological evaluation of the migration extract of an organomodified clay nanocomposite (PLA-Clay1). For this purpose, a histopathological study was performed by optical and electron microscopy in the main organs of Wistar rats after 90 days of oral exposure. Moreover, clinical biochemistry, inflammation and oxidative stress biomarkers were also investigated.

Materials and Methods

Supplies and Chemicals

General chemicals were provided by Sigma Aldrich (Madrid, Spain) and VWR International Eurolab (Barcelona, Spain).

Nanocomposite synthesis and migration extract

Poly (lactic) acid nanocomposite was obtained by ITENE with the modified clay, Clay1. This was prepared by a cation-exchange method, which consists on a displacement of the sodium cations of MMT with the ammonium cations of the hexadecyltrimethyl-ammonium bromide (HDTA) salt. PLA pellets were blended with 4% in weight of Clay1 as described by Jordá-Beneyto et al. (2014).

Migration test was carried out according to UNE-EN 1186-9:2002 (AENOR, 2002) as described by Maisanaba et al. (2014a). Samples, bottles of 150 mL of nanocomposite material PLA+ Clay 1 (123,655 dm^2 and 590-598 µm thick) were filled with the simulant chosen according to the law (EU Commission Regulation N° 10/2011 and Council Directive 82/711/EEC), distilled water, at the specific conditions established by the regulations (Temperature (°C): 40; Time (days): 10).

Animals and Experimental Design

A repeated dose 90-day oral toxicity study was performed in rats. Briefly, 20 male Wistar rats provided by Janvier S.A.S (France) with a mean weight of 239±1 g were used. Rats were fed during the acclimation week with a standard laboratory diet (Harlan 2014, Harlan Laboratories, Barcelona, Spain), and water, both *ad libitum*, in a controlled-temperature room (23 ± 1ºC) with 12 h dark/light cycle, and free from any source of chemical contamination. After this period, animals were randomly divided in two groups, a control group (n=10) and an exposed group (n=10). Both groups were fed with standard diet; the control group received distilled water as beverage while the exposed group was administered with PLA-Clay1 extract as beverage. Glass bottles were used in both groups along the trial to avoid interferences. During the treatment

period clinical signs, body weight, and food and water/extract consumption were recorded weekly.

All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes (Directive 2010/63/EU) and all the procedures were previously accepted by the Ethic Committee of the University of Seville.

Organs and blood sampling

At the end of the experimental period, rats were fasted for 18 h before sacrifice. Liver, kidneys, lungs, spleen, brain, testes, gastrointestinal (g.i.) tract and heart were excised, rinsed with cold saline solution and weighted. Blood samples were obtained by cardiac punction and collected in test tubes with/without lithium heparin depending on the experiment. Serum was separated by low speed centrifugation at 1500 x g at 4ºC for 15 min, and stored at -80ºC until analysis of IL-6 leakage and clinical biochemistry parameters. Liver and kidney sections were homogenized using 50mM phosphate monobasic, 1mM tetrasodium dihydrate salt and bovine serum albumin (BSA) buffer (pH 7,5) at 0-4 ºC with a polytron homogenizer (Heidolph RZR2102 Control, Sigma, Madrid, Spain). The homogenates were centrifuged 20 min at 4000 rpm at 4°C. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for enzyme measurements.

Histopathogical analysis

Tissue samples for histological examination were taken from liver, kidneys, lungs, spleen, brain, testes, intestine and heart of control and exposed rats. For light

microscopy, samples were first fixed in 10% buffered formalin for 24 h at 4ºC, and then immediately dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax using an automatic processor. Sections of 3-5 mm were mounted. After they had been deparaffinized, sections were rehydrated, stained with haematoxylin adenosin (HE), and mounted with Crystal/Mount (Paraplast, Oxford Labware, St. Louis, MO). Liver tissue sections were also stained with periodic acid Schiff (PAS) (Sigma-Aldrich, Madrid, Spain) for glycogen content assessment. For electron microscopy (EM), samples were prefixed in 2% glutaraldehyde fixative (in pH 7.4 phosphate buffer for 10 h at 4ºC) and postfixed in 1% osmium tetroxide fixative (in pH 7.4 phosphate buffer for 30 min at 4ºC). Subsequently, they were dehydrated in a graded ethanol series and embedded in epon. Ultrathin sections, 50–60 nm, were cut with a LKB microtome. The sections were mounted on a copper grid and stained with uranylacetate and lead citrate. The tissue sections were examined in a Philips CM10 electron microscope (FEI, Eindhoven, The Netherlands).

IL-6 leackage

For this assay the blood serum of experimental animals were used. Manufacturer instructions from the kit (Thermo Scientific Rat Interleukin-6 (IL-6) ELISA) were followed.

Oxidative stress biomarkers

GSH/GSSG levels

The ratio GSH/GSSG was determined in blood samples using a commercial kit (Bioxytech GSH/GSSG- 412; Oxis Research, Foster City, CA, USA).

Lipid peroxidation

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman 1990). Malondialdehyde (MDA) was formed as a lipid peroxidation end-product that reacts with TBA reagent under acidic conditions to generate a pink coloured product. Values were expressed as nmol of thiobarbituric acid reactive substances (TBARs)/ g tissue.

Antioxidant enzymes activities

Catalase (Enzyme Commission [EC] 1.11.1.6) activity was assayed in liver and renal homogenates following the method described by Beers and Sizer (1995), measuring the initial rate of H_2O_2 (10 mM) decomposition at 240 nm. Total superoxide dismutase (EC 1.15.1.1) activity was determined based on the method described by McCord and Fridovich (1969). Glutathione peroxidase (EC 1.11.1.9) activity was assayed by following the rate of reduced nicotinamide adenine dinucleotide phosphate oxidation at 340 nm by the coupled reaction with glutathione reductase. The specific activity was determined using the extinction coefficient of 6.22/mM/cm (Lawrence and Burk, 1976). Glutathione S-transferase activity (EC 2.5.1.18) was measured according to the method described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene as a substrate. All enzymatic activities are expressed in nKat/mg protein. Protein content in the samples was estimated by the method of Bradford (1976).

Genetic expression analysis

RNA preparation and reverse transcription

Total RNA was extracted and purified using the RNAeasy Mini Kit™ (Qiagen, Madrid, Spain) according to the manufacturer's instructions. The RNA integrity was

assessed by agarose gel electrophoresis. RNA quality was assessed from the 260/280 nm absorbance ratio using a NanoDrop 2000 (Thermo Scientific, USA). The RNA was then stored at 80ºC. The RNA transcription into cDNA was carried out with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions. The cDNA was stored at -20ºC until further use.

Reverse transcription and real-time polymerase chain reaction (RT-PCR) for CAT and SOD

A semi-quantitative RT-PCR protocol was developed to measure the mRNA levels of SOD and CAT in liver and kidney, using GAPDH as protein control, as described in Maisanaba et al. (2014b). The forward and reverse primers used in this study are shown in Table 1. All mRNA sequences for SOD, CAT and GAPDH were obtained from GenBank. PCR primers were obtained from Sigma-Aldrich (Madrid, Spain). The quantitative fold changes in mRNA expression were determined relative to the GAPDH mRNA levels in each corresponding group and calculated using the 2–ΔΔCTmethod (Gutiérrez-Praena et al., 2013).

Western blot analysis for CAT

Liver and kidney tissues were prepared for the measurement of CAT abundance by a Western blot analysis. Liver and kidney homogenized samples were mixed (1:1) with sample buffer containing β-mercaptoethanol (5%). Proteins were electrotransferred onto nitrocellulose membranes and inmunoblotted as described by Gutiérrez-Praena et al. (2013) and Maisanaba et al. (2014b) using monoclonal anti-CAT antibodies (Sigma-Aldrich, Madrid, Spain), diluted at 1/10000. The anti-CAT was detected by the enhanced chemiluminiscence (ECL) method according to the supplier´s protocol

(Sigma, Madrid, Spain) and using a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, USA) as secondary antibody (1/10000).

Statistical analysis

Data are expressed as mean ± standard deviation of ten animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

Results

No rat died during the experimental period and there were no remarkable clinical signs. Body weight, body weight gain (Fig. 1), food and water/extract consumption (Fig. 2), organs weight and the somatic index of the different organs (Table 2) did not show statistical differences between the control and the exposed group.

Histopathogical results

In general, histopathological results from rats exposed to the extract did not show significant variations respect to the control group. More specifically, when the microscopic examination of the HE-stained liver section was carried out, no differences appeared between the exposed group (Fig. 3C) and the control group (Fig. 3A). Thus, hepatocytes from the parenchyma appeared with a normal conformation and normally disposed in hepatic cords. Furthermore, nuclei of the hepatocytes are shown well centered and the cytoplasm was clear. Ultrastructurally, hepatocytes from both control and exposed groups appeared with centered nuclei, numerous organoids, and amorphous glycogen (Figs. 3B, 3D).

Kidneys presented a normal parenchyma in the control group (Fig. 4A) as well as in the exposed group (Fig. 4C). They showed well disposed glomeruli. The Bowman's capsule presented a regular disposition, as well as the distal and the proximal convoluted tubules. Under electronic microscopy, fenestrated capillary, podocytes, and pedicels from both groups appeared without any sign of damage (Figs. 4B, 4D).

Regarding to the microscopic examination of the HE-stained lung sections of both experimental groups, the parenchyma did not show any alteration. Thus, bronchioles and alveolar sacculations were easily observable (Figs. 5A, 5C). The electron microscopy images showed the presence of pneumocytes type I covering the alveolar lumen, as well as intra-alveolar macrophages with abundant lysosomes (Figs. 5B, 5D).

Spleen also showed a normal structural conformation under light microscopy in both groups. The white pulp was perfectly identified, as well as other inside structures such as lymphoid follicles, crossed by the central artery (Figs. 6A, 6C). When studying the images obtained from electron microscopy, lymphoid follicles showed abundant lymphocytes and reticular cells (Figs. 6B, 6D).

Concerning to the brain, no observable damage appeared in the neural tissue in any of the groups. In fact, light and the electron microscopy studies showed the same cellular structures. That is, normal neuronal bodies with rounded nuclei as well as the neuropiles (Fig. 7).

Testes did not show remarkable variations in their parenchyma in the control group, not even in the exposed group. Under light microscopy, both seminiferous tubules and interstitial tissue were well disposed (Figs. 8A, 8C). Ultrastructurally, a type B spermatogonium was evidenced in the control group (Fig. 8B), and also a type A spermatogonium in the exposed group (Fig. 8D). Both processes correspond to a normal status of the testes.

The gastrointestinal tract presented unaltered villi with abundant enterocytes, in both experimental groups (Figs. 9A, 9C). At ultrastructural level, enterocytes presented well developed microvilli. Unaffected caliciform cells were also observed (Figs. 9B, 9D).

Finally, heart was apparently normal in the two studied groups. Under light microscopy, cardiac fibers presented uniformed size and morphology. Images showed the cardiac fibers longitudinally cut (Fig. 10A) or perpendicularly cut (Fig. 10C). The electron microscopy study revealed that the cardiac fibers presented well centered nuclei, and apparently normal myofibrils and desmosomes union zones were also observable (Figs. 10B, 10D).

Clinic Biochemistry Parameters

Results showed no significant changes in any of them in comparison to the control group (Table 3).

IL-6 leackage

No IL-6 leakage was observed in the serum of any of the experimental groups (data not shown).

Oxidative stress biomarkers

GSH/GSSG levels and Lipid peroxidation

Blood GSH/GSSG ratio did not experience a significant alteration in rats exposed to PLA-Clay1 extract in comparison to the control group (Fig. 11). Similarly, lipid peroxidation was altered neither in liver nor in kidney (Fig. 12).

Antioxidant enzymes activities

CAT and SOD activities were not affected by the PLA-Clay1 exposure in any of the organs studied (Fig. 13). In a similar way, GPx and GST activity did not show any change after 90 days of exposure (Fig. 14).

Genetic expression analysis

CAT and SOD gene expression showed the same pattern that their activities, with no noticeable changes in the exposed group in comparison to the control group (Fig. 15).

Western blot analysis for CAT

The protein abundance of CAT in the liver and the kidney were not altered by the treatment (Fig. 16).

Discussion

The incorporation of nanoparticles to food products is of concern due to the potential exposure of consumers and the scarce toxicological data available about this kind of compounds. In the present study, the subchronic oral exposure of rats to the migration extract of a PLA-Clay1 nanocomposite did not derive in observable adverse effects in any of the biomarkers studied. Clay1 is a no commercially available organoclay developed by ITENE. A previous *in vitro* evaluation showed that Clay1 did not produce cytotoxicity on the intestinal human cell line Caco-2 up to 8 µg/mL. However, a significant decrease of viability at this concentration in the human hepatocellular cell line Hep-G2 measured by the neutral red uptake assay was observed (Jordá-Beneyto et al., 2014). Higher Clay1 concentrations could not be assayed in vitro due to its hydrophobicity resulting in interferences with the spectrophotometic measurements. Similarly, other organoclays have shown to induce toxic effects in vitro (Sharma et al., 2010; Lordan et al., 2011; Baek et al., 2012; Maisanaba et al., 2013a, b) including genotoxicity and oxidative stress. Others, however, have not shown cytotoxic effects (Maisanaba et al., 2013a, b; Houtman et al., 2014) suggesting that a case by case evaluation is needed.

Moreover, it is also important to evaluate the toxicity in animal models, since *in vitro* cell culture systems does not fully mimic the complicated *in vivo* system, being devoid of immune systems, diverse protein reactions, and dynamic fluidic environments (Baek et al., 2012). In this sense, Wistar rats exposed to 40 mg/kg/day Clay1 in the diet for 90 days did not show histophatological damages (Maisanaba et al., 2014c). Moreover, they did not find changes in the clinic biochemistry parameters and inflammatory biomarkers. Among oxidative stress biomarkers, however, they observed a significant increase in the activity, genetic expression and protein abundance of

catalase in the kidney, an enzyme involved in the detoxification of reactive oxygen species (ROS) (Maisanaba et al., 2014b). Apart from the aforementioned study, toxicological oral *in vivo* experiments with clays are still scarce in the scientific literature and most of them are related to acute exposures. Thus, Baek et al., (2012) exposed mice to a single dose of up to 1000 mg/kg MMT by gavage, not evidencing any mortality or remarkable abnormal behavior or symptom 14 days after exposure. Similarly, male Wistar rats exposed to 450 mg/kg of different clays for 3 days did not show any macro-toxic effects, although they the ingestion of clays caused an increase of metals in the urine and organs (kidney>liver>heart>brain) that could be toxicologically relevant (Mascolo et al., 1999, 2004).

In addition, despite little is known about the toxicological profile of bulk clays the toxicity of nanoclays is a different concern, since this cannot be inferred from their bulk counterparts. In this sense, clays have a size in the micrometer range and when they are imbued in the polymeric matrix they split up in platelets with a nanometric thick, constituting a nanocomposite. This makes the toxicological evaluation of these new products more difficult to perform. Li et al. (2010) evaluated the cytotoxicity and genotoxicity of exfoliated silica plates with a thickness of 1 nm obtained from MMT. They observed a reduction in cell viability from 62.5 µg/mL in CHO cells, no DNA damage by the Comet assay, no chromosomal damages in the micronucleus assay and no mutagenicity in five different *Salmonella typhimurium* strains. Moreover, they did not observe any mortality, clinical signs or macroscopic changes in an acute oral toxicity in rats. On the contrary, Yoshida et al. (2012) found that amorphous nanosilica particles (external diameter, 70 nm) induced cytotoxicity, ROS generation and DNA

damage in various mammalian cells and that these toxic effects were reduced by surface modification.

In the present work, the toxicological evaluation of the migration extract from a PLA-Clay1 nanocomposite has been performed. This represents a more real exposure scenario because consumers would be potentially exposed to the migrants. A preliminary characterization performed on the PLA-Clay1 extract by Maisanaba et al. (2014a), indicated no significant migration of the main metallic elements present in Clay1 (Al, Ca, Mg, Fe, Si). However, a fully analytical determination, including the study of nanosilica platelets in the extract, has been not performed. Actually, the European Food Safety Authority (EFSA) (2011) recognizes that currently it is not possible to routinely determine engineered nanomaterials *in situ* in the food. The absence of toxic effects observed is in agreement with the scarce presence of the migrants analyzed. Also, the changes observed in the activity, genetic expression and protein abundance of CAT enzyme in rats exposed to Clay1 are suppressed with the PLA-Clay1 exposure. The PLA-Clay1 extract have also shown that it does not induce cytotoxicity in Caco-2 and HepG2 cell lines (Maisanaba et al., 2014a) in comparison to Clay1 (Jordá-Beneyto et al., 2014, Houtman et al., 2014). Also, the PLA-Clay1 extract has demonstrated to induce no mutagenic effects by the Ames test (Maisanaba et al., 2014a).

Conclusion

Results of the present work suggest that, regarding safety issues, PLA-Clay1 nanocomposite has a good profile to be used in the food packaging industry, however further studies are needed to confirm this hypothesis.

Acknowledgments

The authors wish to thank Ministerio de Ciencia e Innovación (AGL2010-21210) and Junta de Andalucía (AGR5969) for the financial support for this study.

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Figures legends

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Figure 2. Water/extract (a) and food consumption (b) of control Wistar rats and rats exposed to PLA-Clay1 extract as beverage for 90 days. Results are expressed as mean ± sd.

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Figure 5. Histopathological changes in lungs of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained lung section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Pulmonary parenchyma with normal alveoli (AI). B. Detail of pulmonary alveoli with long pneumocytes type I (NI) covering the alveoli (Al). C, D: Exposed rats. C. Pulmonary parenchyma with normal alveoli (Al) and bronchi (Br). D. Detail of pulmonary alveoli.

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Figure 8. Histopathological changes in the testes of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained testes section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Detail of testicular parenchyma with seminiferous tubules (St) and interstitial tissue (Int). B. Detail of seminiferous tubule with spermatogonia (G). C, D: Exposed rats. C. Detail of testicular parenchyma with seminiferous tubules (St) and interstitial tissue (Int). D. Detail of seminiferous tubule with plenty of spermatids (Sd).

Figure 9. Histopathological changes in the intestine of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained intestine section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Normal intestinal villi (V) and normal enterocytes (En). B. Enterocytes (En) with highly developed microvilli (Mi) with normal appearance. C, D: Exposed rats. C. Normal intestinal villi (V) and normal enterocytes (En). D. Enterocytes (En) with highly developed microvilli (Mi) with normal appearance.

Figure 10. Histopathological changes in the heart of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained heart section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Normal cardiac parenchyma with cardiac fibers (Cf) in a longitudinal section. B. Detail of a cardiac fiber (Cf), with central nuclei and apparently normal myofibrils (Mi). C, D: Exposed

rats. C. Normal cardiac parenchyma with cardiac fibers (Cf) in a transversal section. D. Detail of a cardiac fiber (Cf), with central nuclei and apparently normal myofibrils (Mi).

Figure 11. Blood GSH/GSSG ratio in control Wistar rats and rats exposed to PLA-Clay1 extract for 90 days. Results are expressed as mean ± s.d.

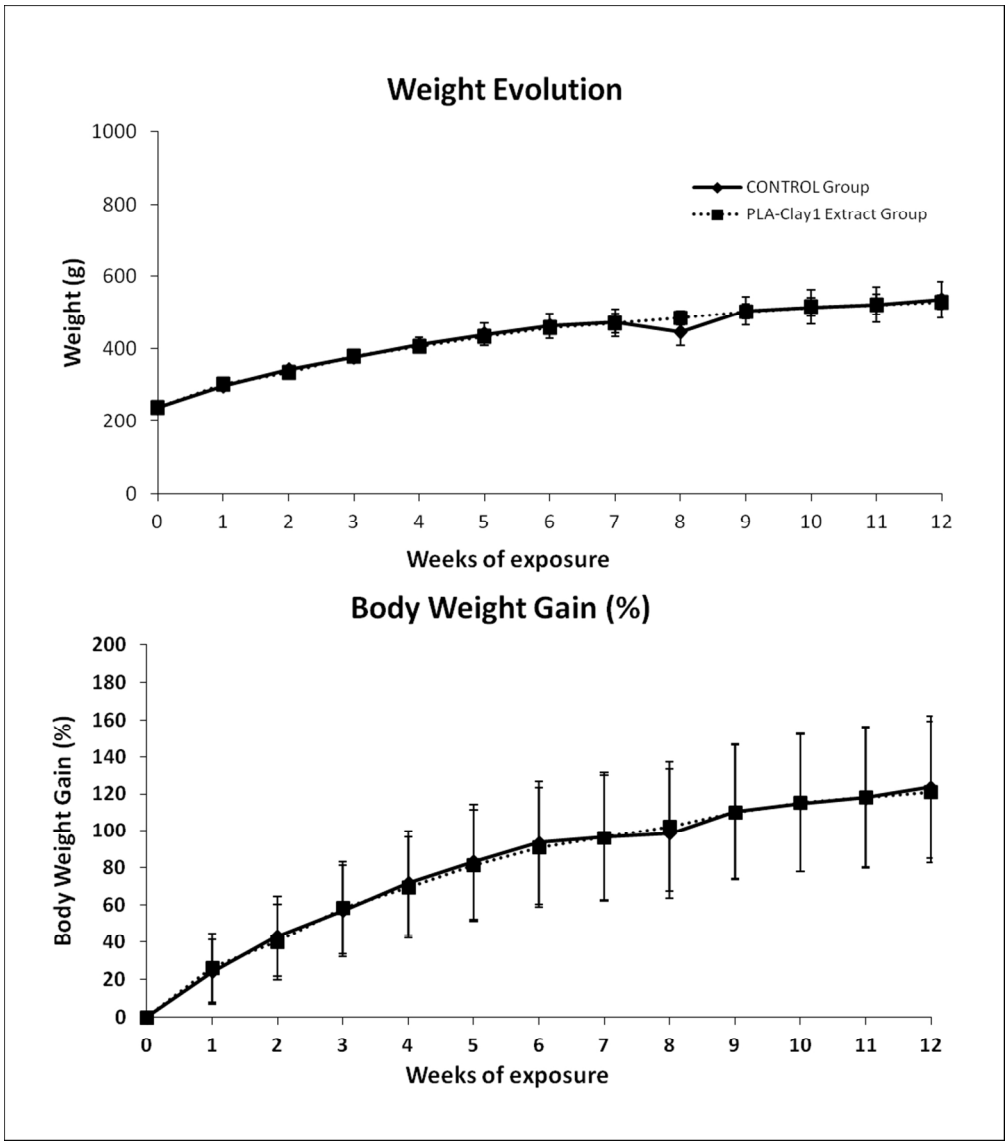
Figure 12. Effect of PLA-Clay1 extract on lipid peroxidation on liver (a) and kidney (b) of rat. The values are expressed as mean±S.D. (n=10). LPO value is expressed as nmol TBARs/g tissue.

Figure 13. Catalase (CAT) and superoxide dismutase (SOD) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).

Figure 14. Glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).

Figure 15. Relative gene expression of catalase (CAT) and superoxide dismutase (SOD) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).

Figure 16. Relative abundance of catalase (CAT) protein in liver (a) and kidney (b) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).



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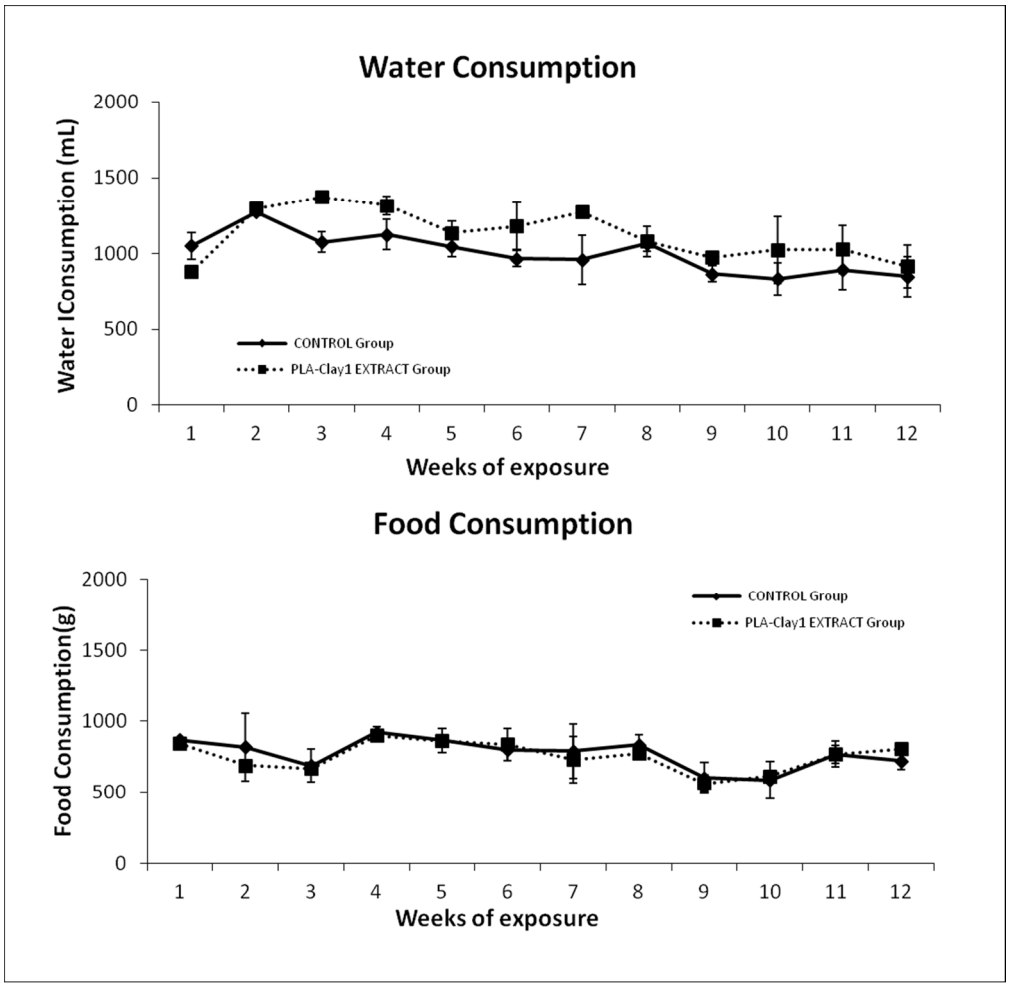

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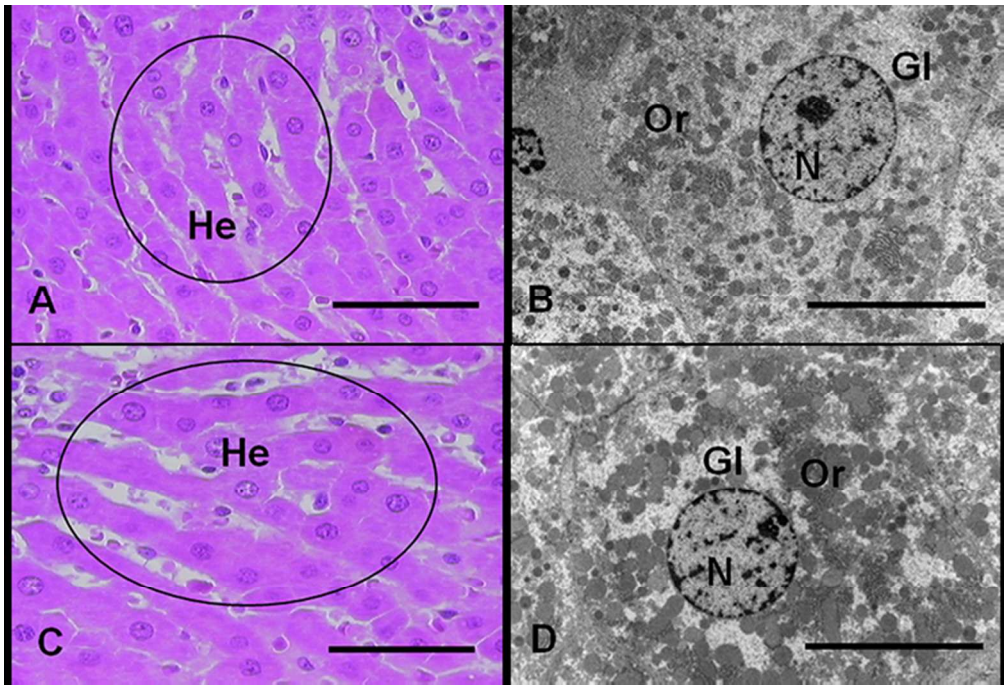

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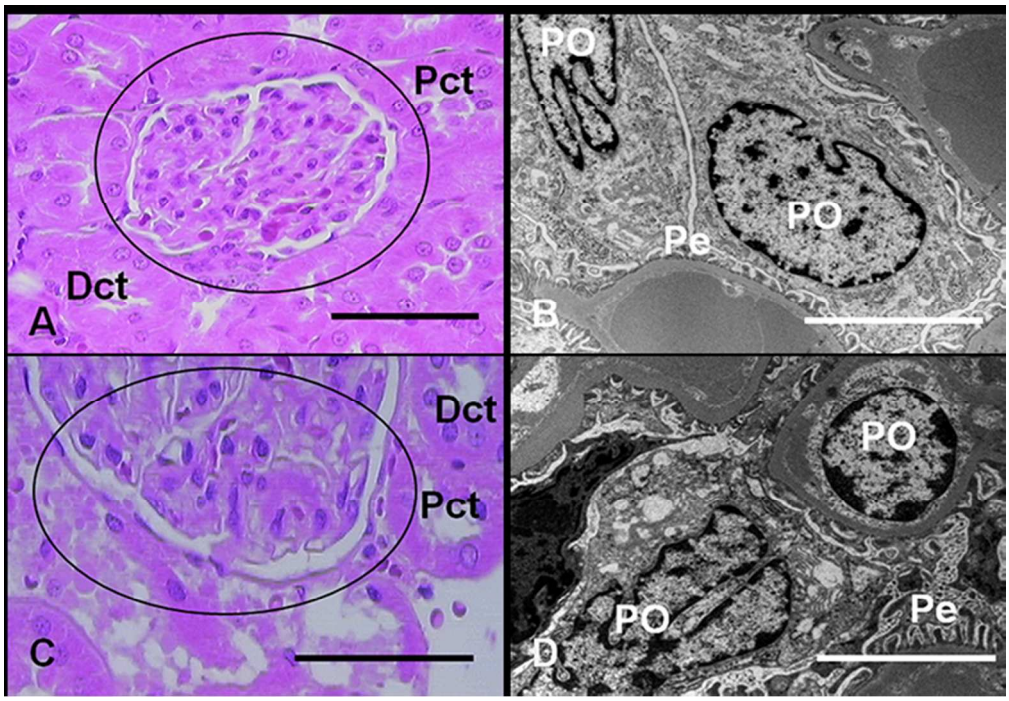

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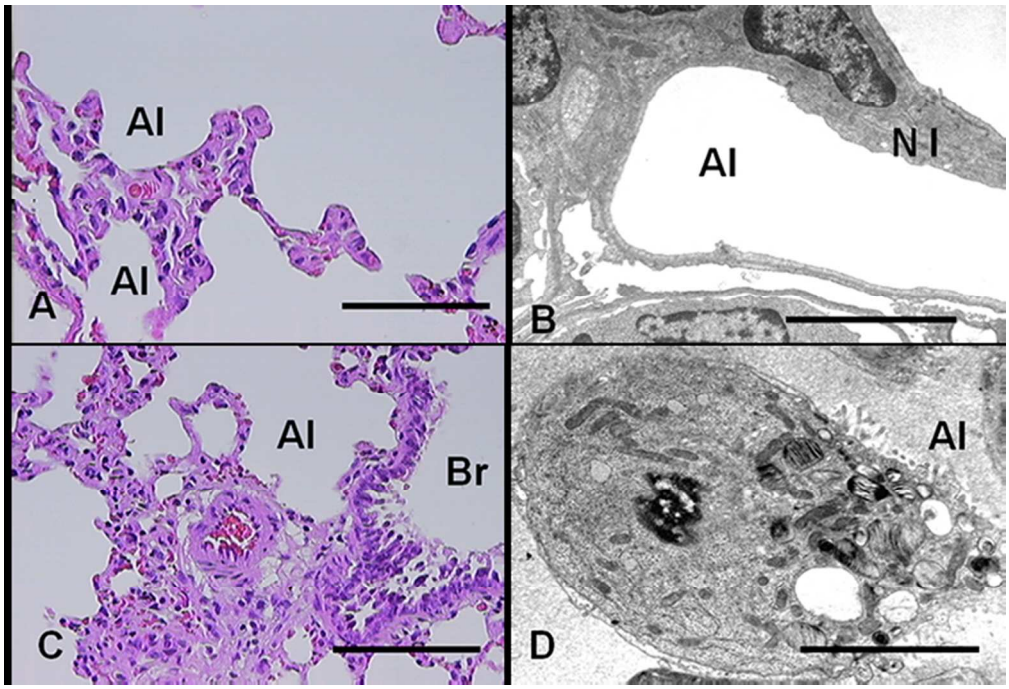

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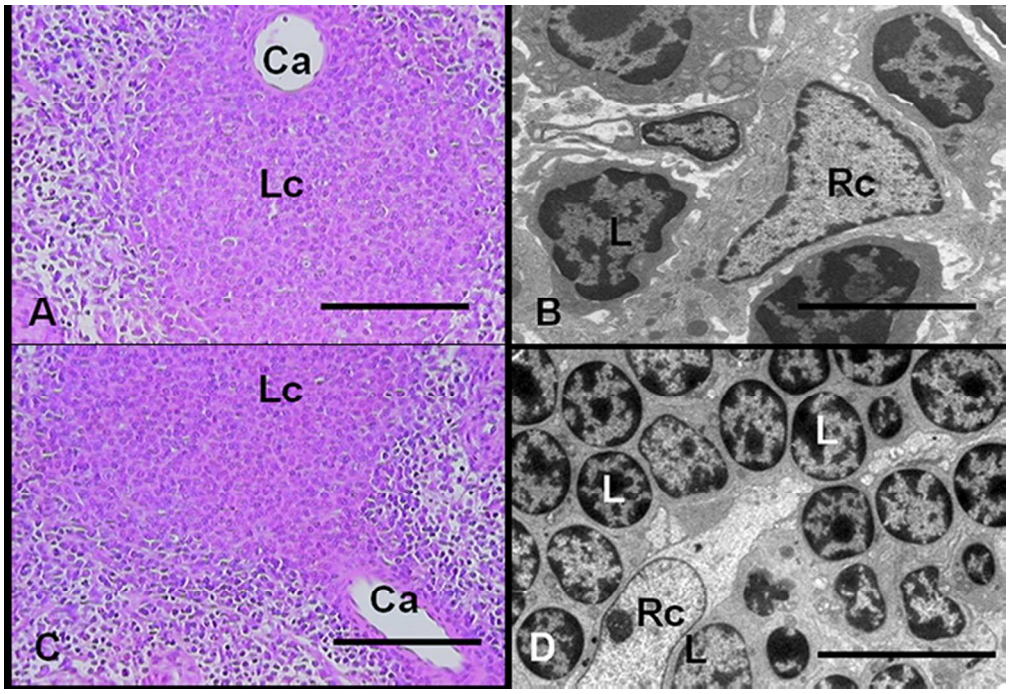

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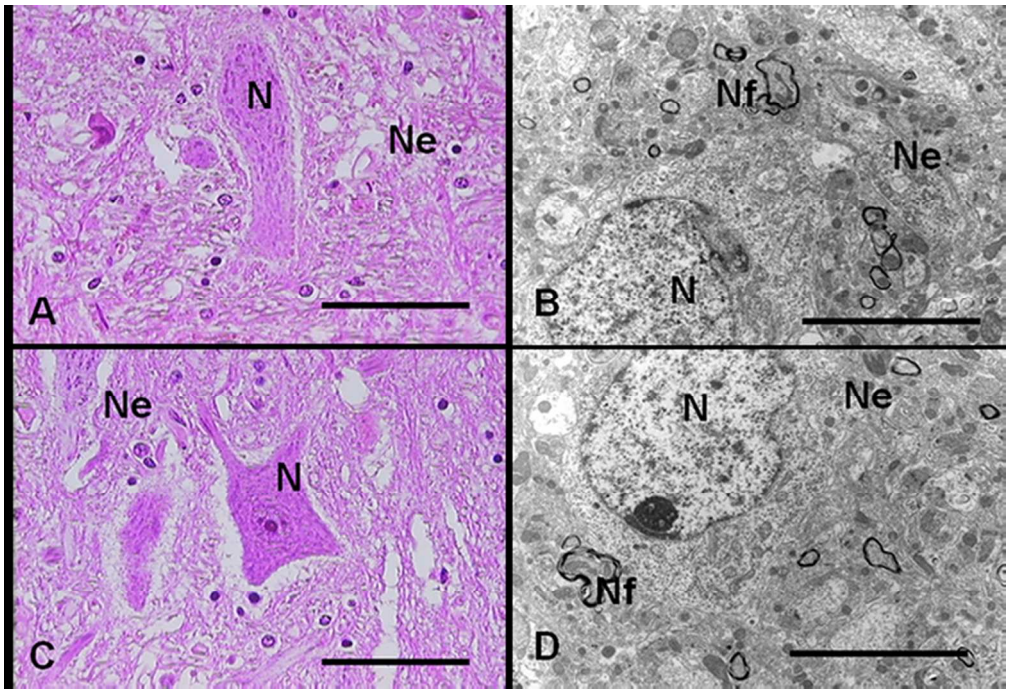

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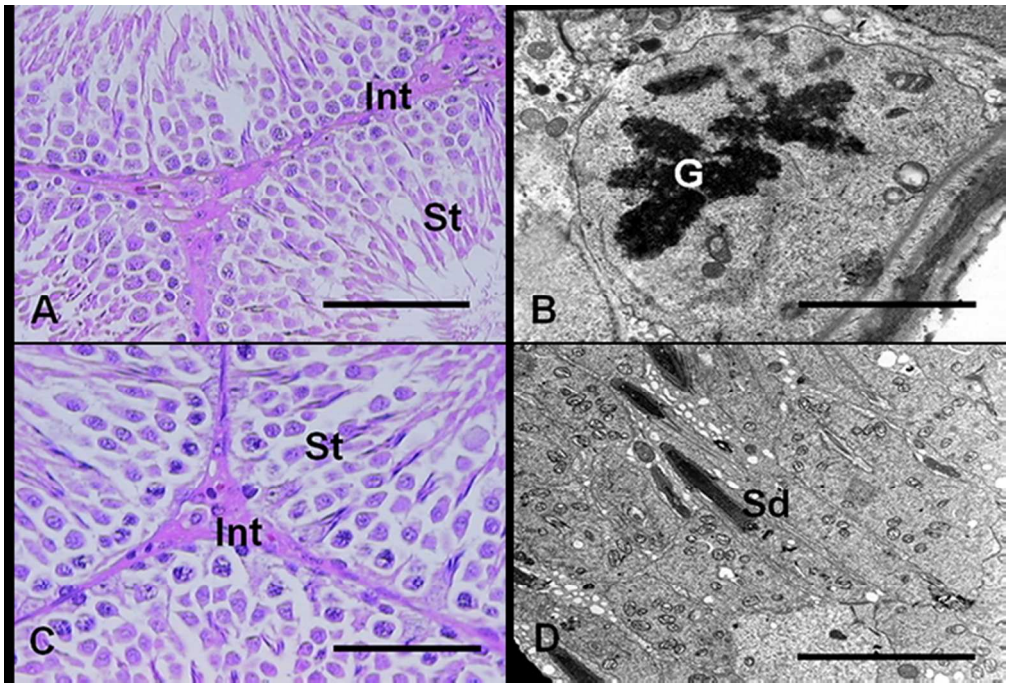

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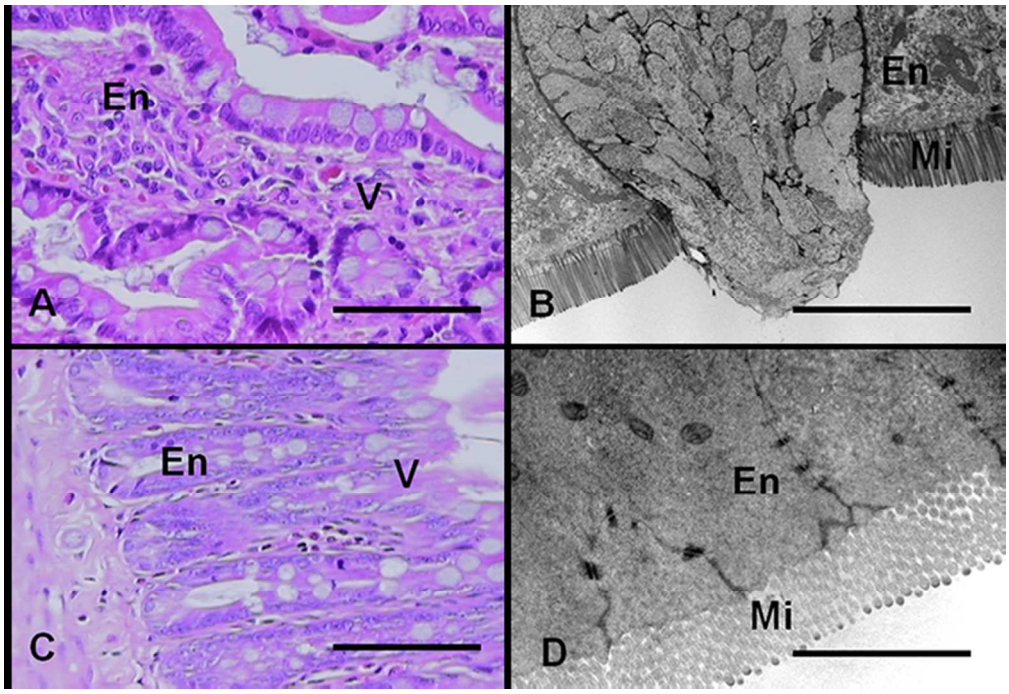

Figure 9. Histopathological changes in the intestine of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained intestine section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Normal intestinal villi (V) and normal enterocytes (En). B. Enterocytes (En) with highly developed microvilli (Mi) with normal appearance. C, D: Exposed rats. C. Normal intestinal villi (V) and normal enterocytes (En). D. Enterocytes (En) with highly developed microvilli (Mi) with normal appearance.
61x42mm (300 x 300 DPI)

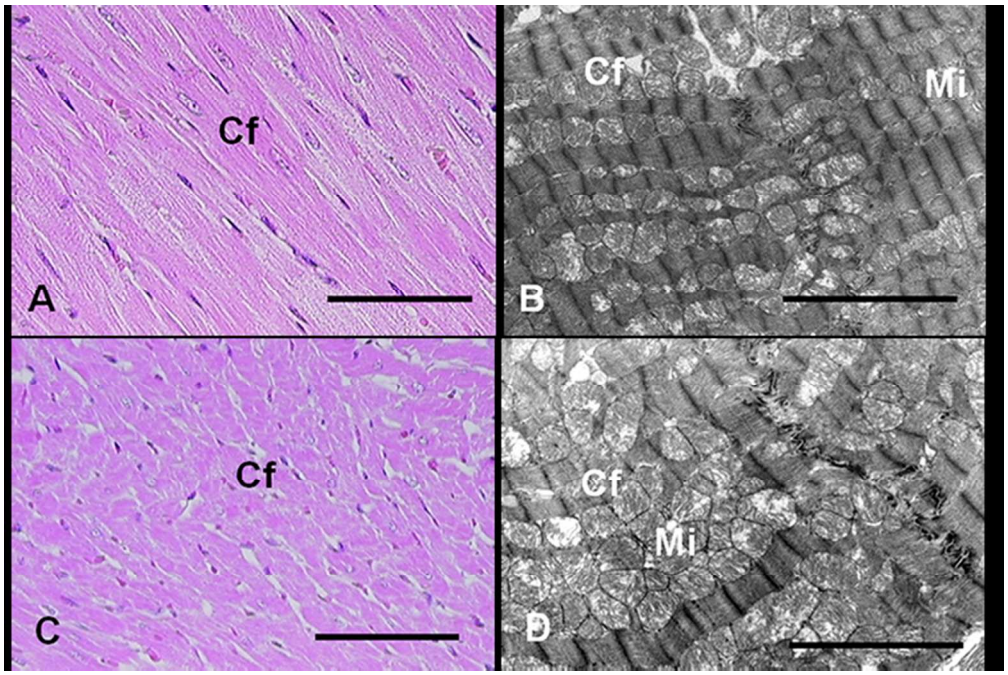

Figure 10. Histopathological changes in the heart of Wistar rats exposed to a PLA-Clay1 extract as beverage for 90 days. (A, C): HE-stained heart section. Bars, 100 µm. (B, D): Ultrastructural observations. Bars, 10 µm. A, B: Control rats. A. Normal cardiac parenchyma with cardiac fibers (Cf) in a longitudinal section. B. Detail of a cardiac fiber (Cf), with central nuclei and apparently normal myofibrils (Mi). C, D: Exposed rats. C. Normal cardiac parenchyma with cardiac fibers (Cf) in a transversal section. D. Detail of a cardiac fiber (Cf), with central nuclei and apparently normal myofibrils (Mi).
63x42mm (300 x 300 DPI)

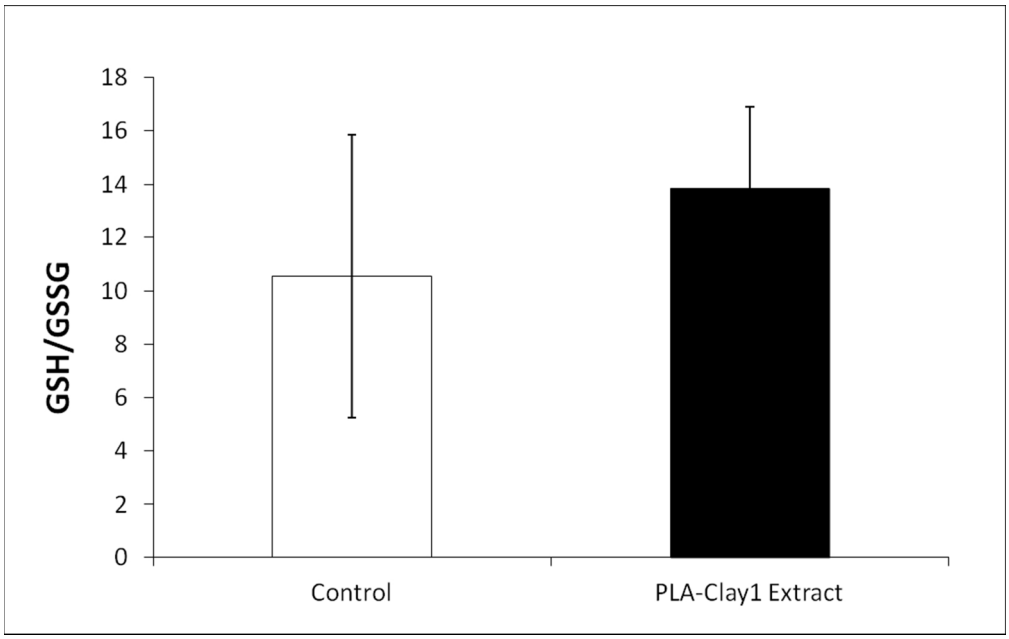

Figure 11. Blood GSH/GSSG ratio in control Wistar rats and rats exposed to PLA-Clay1 extract for 90 days. Results are expressed as mean ± s.d.
182x114mm (300 x 300 DPI)

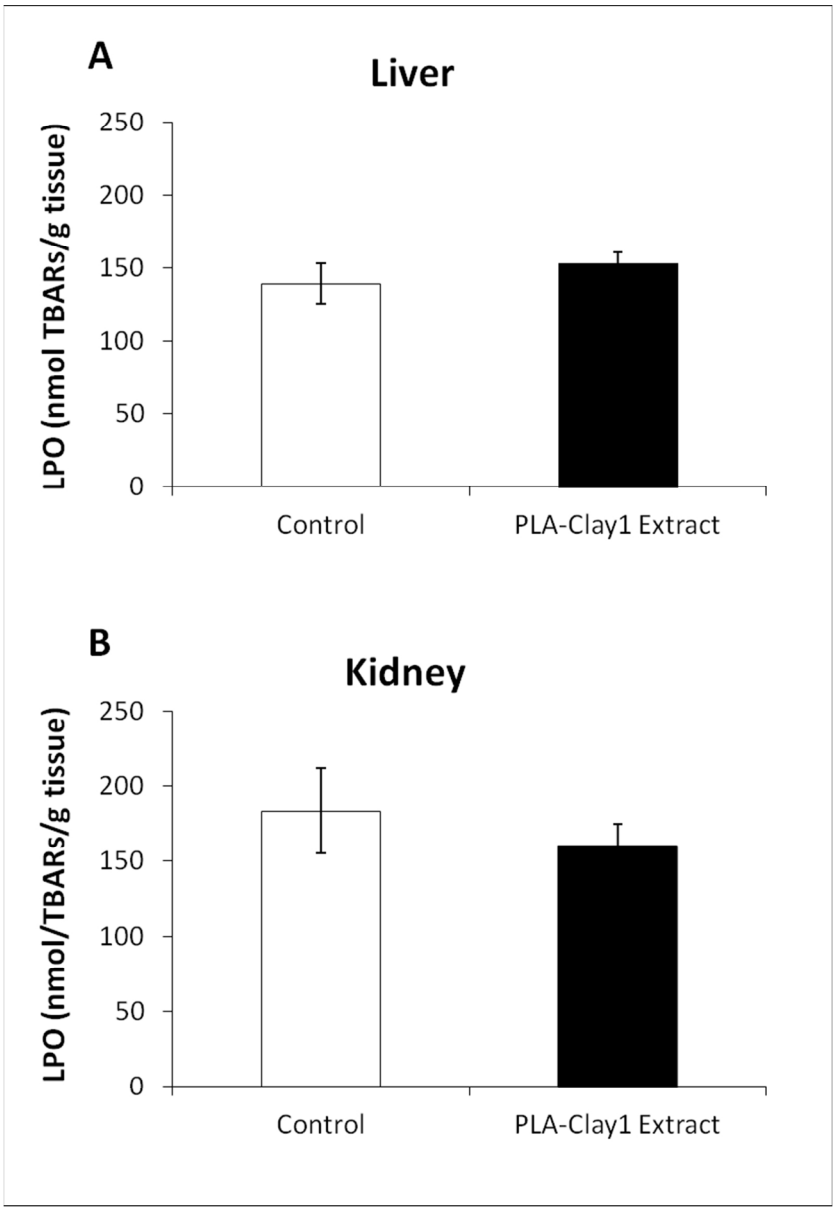

Figure 12. Effect of PLA-Clay1 extract on lipid peroxidation on liver (a) and kidney (b) of rat. The values are expressed as mean±S.D. (n=10). LPO value is expressed as nmol TBARs/g tissue.
138x200mm (300 x 300 DPI)

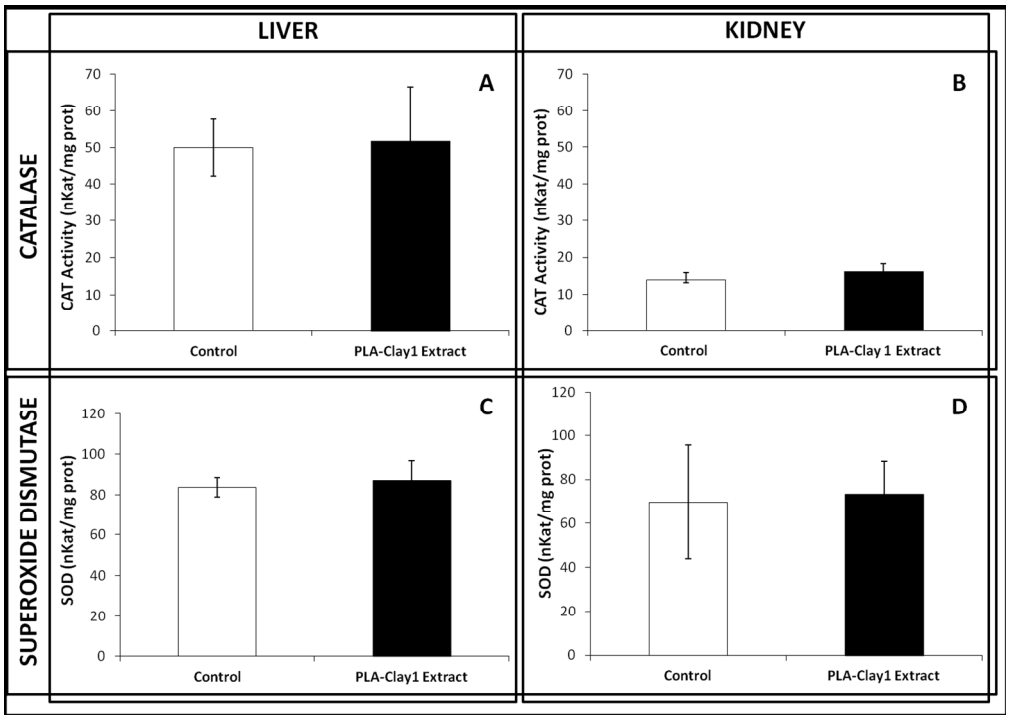

Figure 13. Catalase (CAT) and superoxide dismutase (SOD) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).
270x191mm (300 x 300 DPI)

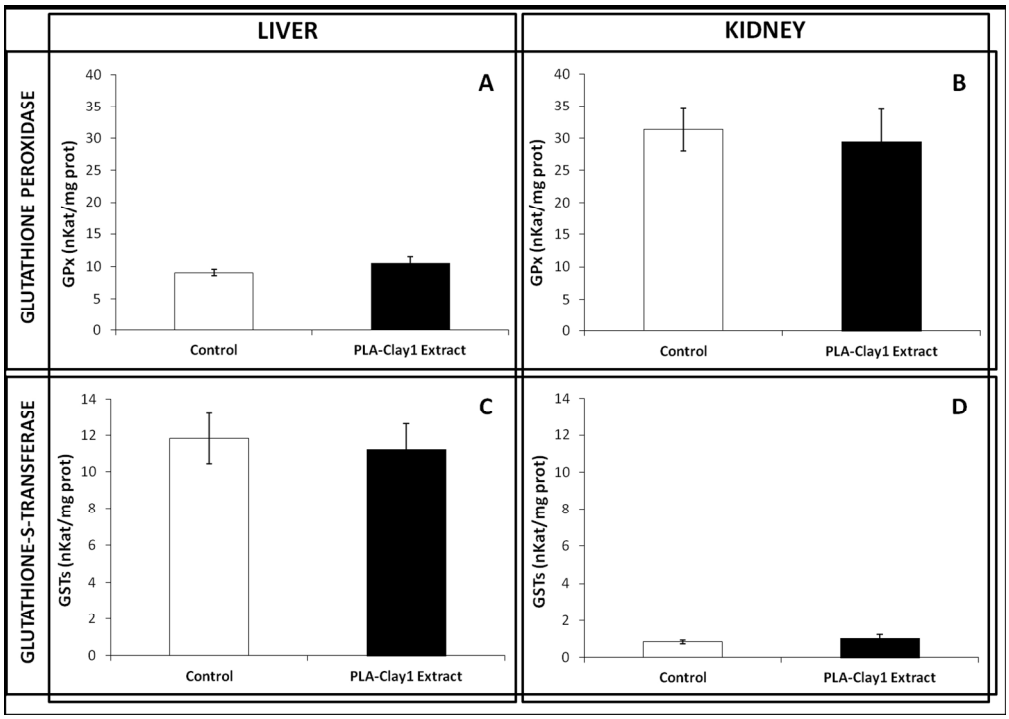

Figure 14. Glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities (nkat/mg protein) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).
270x191mm (300 x 300 DPI)

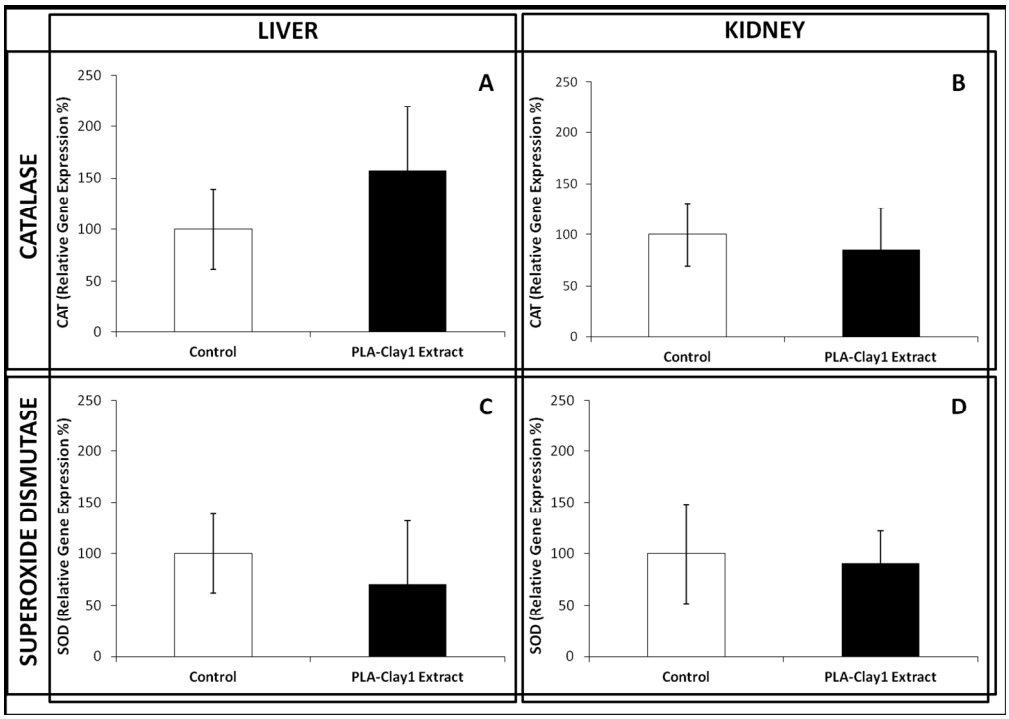

Figure 15. Relative gene expression of catalase (CAT) and superoxide dismutase (SOD) in liver (a,c) and kidney (b,d) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).
271x191mm (300 x 300 DPI)

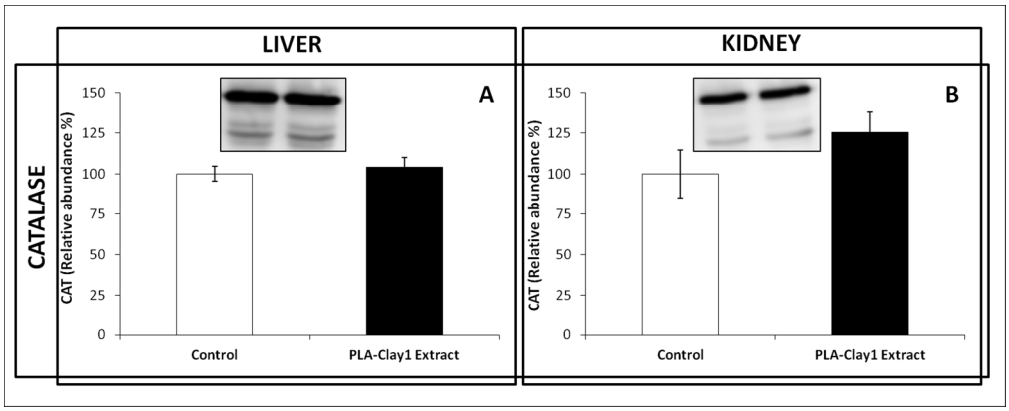

Figure 16. Relative abundance of catalase (CAT) protein in liver (a) and kidney (b) of rat exposed to PLA-Clay1 extract. The values are expressed as mean±sd. (n=10).
275x110mm (300 x 300 DPI)

Table 1. Nucleotides sequences (5'-3') of PCR primers of GAPDH, SOD, and CAT.

Gene*	Forward primer	Reverse primer
GAPDH	GCCAAAAGGGTCATCATCTCCGC	GGATGACCTTGCCCACAGCCTTG
SOD	CGTCATTCACTTCGAGCAGAAGG	GTCTGAGACTCAGACCACATA
CAT	CCTTTATCCATAGCCAGAAG	AGCGTGAGTCTGCGCTTCAG

* GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SOD: Superoxide dismutase; CAT: Catalase

Table 2. Organs weight (g) and somatic index (%) of the different organs of control Wistar rats and rats exposed to PLA-Clay1 extract for 90 days. Results are expressed as mean ± sd.

	Control	**PLA-Clay1 Extract**
	Mean ± SD	Mean ± SD
Liver (g)	13.12 ± 2.44	12.43±0.83
Somatic Liver Index	2.83 ± 0.40	2.41±0.14
Kidneys (g)	3.02 ± 0.63	2.91±0.22
Somatic Kidney Index	0.65 ±0.11	0.57±0.05
Lungs (g)	2.75 ± 0.78	2.84±0.39
Somatic Lung Index	0.6 ± 0.09	0.56±0.10
Spleen (g)	1.02 ± 0.17	1.10±0.14
Somatic Splenic Index	0.22 ± 0.02	0.21±0.02
Brain (g)	2.06 ± 0.18	2.25±0.08
Somatic Brain Index	0.45 ± 0.05	0.44±0.03
Testes (g)	3.66 ± 0.75	4.22±0.90
Somatic Testicle Index	0.79 ± 0.11	0.80±0.19
Intestine (g)	4.99 ± 1.02	5.27±2.77
Somatic Intestine Index	1.10 ± 0.37	1.00±0.49
Heart (g)	1.82 ± 0.12	1.78±0.20
Somatic Heart Index	0.40 ± 0.05	0.34±0.04

Table 3. Clinic biochemistry parameters of control Wistar rats and rats exposed to PLA-Clay1 extract for 90 days. Results are expressed as mean ± sd.

Parameters	Control Mean ± SD	PLA-Clay1 EXTRACT Mean ± SD
Glucose (mg/dL)	141.83 ± 61.95	156.50±64.8
Urea (mg/dL)	58.00 ± 7.95	54.75±7.93
Creatinine (mg/dL)	0.78 ± 0.08	0.78±0.06
Total protein (g/dL)	6.17 ± 0.19	5.97±0.38
Albumin (g/dL)	4.12 ± 0.22	3.90±0.16
Cholesterol (mg/dL)	90.00 ± 15.61	78.00±11.69
Aspartate Aminotransferase (AST) (U/L)	187.83 ± 67.29	136.25±21.61
Alanine Aminotransferase (ALT) (U/L)	52.67 ± 16.28	48.75±3.09
Alkaline Fosfatase (ALP) (U/L)	185.67 ± 54.64	162.50±36.15
Sodium (mEq/L)	152.67 ± 1.21	150.50±1.73
Potassium (mEq/L)	7.68 ± 1.12	7.40±0.91

CAPÍTULO 13/ CHAPTER 13

Maisanaba S, Pichardo S, Jordá M, Aucejo S, Cameán A, Jos A

EVALUACIÓN DE LA SEGURIDAD DE UNA ARCILLA MODIFICADA Y SU EXTRACTO DE MIGRACIÓN EN BAZO DE RATAS WISTAR EXPUESTAS DE FORMA SUBCRÓNICA

Revista de Toxicología 30, 125-130, ***2013***

Rev. Toxicol. (2013) 30: 138-143

Evaluación de la seguridad de una arcilla modificada y su extracto de migración en bazo de ratas Wistar expuestas de forma subcrónica

Maisanaba S[1], Pichardo S[1], Jordá M[2], Aucejo S[2], Cameán A[1], Jos A[1]*

[1]Área de Toxicología, Facultad de Farmacia, Universidad de Sevilla. C/ Profesor García González nº 2, C.P. 41012, Sevilla, España. [2]Área de Envases y Sistemas, Instituto Tecnológico de Envase, Transporte y Logística, ITENE. C/ Albert Einstein 1, C.P. 46980, Paterna, Valencia España.

Recibido 24 de septiembre de 2013 / Aceptado 16 de noviembre de 2013

Resumen: Las ventajas tecnológicas de la incorporación de arcillas modificadas en polímeros para el envasado de alimentos son bien conocidas, pero aún quedan muchas incertidumbres sobre la seguridad de estos materiales. El Instituto Tecnológico del Embalaje, Transporte y Logística ha desarrollado una arcilla, Clay1, modificando una montmorillonita con una sal de amonio cuaternario. Esta organoarcilla, incorporada al polímero (ácido poliláctico), da lugar a un material nanocompuesto, reforzándose así el material de partida. El principal objetivo de este estudio es evaluar la actividad de biomarcadores de estrés oxidativo en bazo de ratas expuestas durante 90 días a Clay1 (40 mg/kg/día) y al extracto de migración obtenido a partir del material nanocompuesto resultante. Los parámetros evaluados fueron la peroxidación lipídica y las actividades enzimáticas superóxido dismutasa y catalasa. Además, se realizó un análisis del contenido en bazo de los metales más característicos que componen la organoarcilla (Al, Ca, Fe, Mg, Si) para comprobar su posible acumulación. En dicho estudio se trabajó con tres grupos de ratas Wistar (n=10): control (comida estándar + agua como bebida), Clay1 (comida estándar mezclada con 40mg/kg/día de arcilla + agua) y extracto de Clay1 (comida estándar + extracto como bebida). Tras el tiempo de exposición los animales se sacrificaron y se extrajo el bazo. De forma general, no se observaron diferencias significativas en ninguno de los parámetros evaluados con respecto al grupo control, por lo que Clay1 muestra un buen perfil toxicológico respecto a los biomarcadores ensayados con vistas a su uso en la industria alimentaria.

Palabras clave: arcilla modificada, envasado de alimentos, exposición subcrónica, estrés oxidativo

Abstract: Influence of a subchronical exposure to a modified clay and its migration extract in the spleen of Wistar rats. The technological advantages of the incorporation of modified clays into polymers for food packaging are well known. However, there are still many uncertainties about the safety of these materials. The Technological Institute of Packaging, Transport and Logistic has developed Clay1, a modified clay with a quaternary ammonium salt. This organoclay is incorporated into the polymer (polylactic acid), giving a nanocomposite material and reinforcing the bulk material. The aim of this study is to evaluate the activity of several oxidative stress biomarkers in the spleen of rats exposed for 90 days to Clay1 (40 mg/kg/day) and its migration extract obtained from the resultant nanocomposite material. The parameters evaluated were lipid peroxidation and superoxide dismutase and catalase activities. Moreover, the characteristic metallic components of the organoclay (Al, Ca, Fe, Mg, Si) were also analyzed to test the possible accumulation. In this study, three groups of Wistar rats (n=10) were used: control (standard food + water), Clay1 (food with Clay1+water) and Clay1 extract (standard food+ Clay1 extract as water). After the exposure the spleen was removed. In general, no significant differences were observed in any of the parameters evaluated compared to the control group, therefore Clay1 showed a good toxicologic profile regarding the biomarkers assayed for its use in the food industry.

Keywords: modified clay, food packaging, subchronical exposure, oxidative stress

Introducción

El uso de arcillas naturales como la montmorillonita (MMT), kaolinita, bentonita, etc., está en auge en diversos campos de trabajo. Por ejemplo, podemos encontrarlas actuando como catalizadores, adsorbentes, en materiales nanocompuestos, en sensores y electrodos, como antibacterianos en determinados materiales, en almacenaje de desechos nucleares y transportadores de pesticidas, etc. [1]. El papel que juegan dentro de la industria alimentaria, y más concretamente en el área de envasado, es cada vez más destacado. El uso de plásticos en el envasado de alimentos está ampliamente instaurado dentro de la industria de la alimentación, pero se debe tener en cuenta que la calidad y seguridad de los alimentos envasados puede verse comprometida si existe la posibilidad de intercambio gaseoso, acuoso y/o de componentes aromáticos [2]. En este aspecto en particular, el uso de las arcillas anteriormente mencionadas cobra un papel importante. La modificación de la superficie de las arcillas minerales recibe una especial atención ya que da lugar a nuevas aplicaciones y a nuevos materiales, siendo destacable el desarrollo de nuevos polímeros nanocompuestos [3]. Las organoarcillas son los nanomateriales más usados en el comercio para preparar estos polímeros nanocompuestos [4]. Así mismo, el aspecto económico es también muy beneficioso, logrando con estos materiales una relación coste-precio-eficiencia notable [5,6].

Entre las arcillas que más destacan para dar lugar a los materiales nanocompuestos se encuentra la MMT sódica, comercialmente conocida como Cloisite®Na^+ (C®Na^+), un hidroxisilicato de magnesio y aluminio, con otros posibles elementos presentes. Tiene una estructura laminar, compuesta por dos láminas tetraédricas formadas por silicio (Si) y oxígeno (O) acoplada a una lámina octaédrica formada por átomos de magnesio (Mg) y aluminio (Al) unidos a O y grupos hidroxilo (OH) [7].

Una vez que la arcilla se imbuye y dispersa en el polímero seleccionado, sus láminas se disponen dando lugar a una estructura que obliga a los gases a seguir una trayectoria tortuosa, consiguiendo de tal forma que se disminuya la permeabilidad. La principal ventaja

*e-mail: angelesjos/us.es

del uso de estas arcillas como "relleno" es la mejora que se produce en las propiedades barrera del polímero [8]. Además, otros autores también destacan las mejoras en las propiedades mecánicas, térmicas, reológicas y ópticas de los materiales nanocompuestos [6,9], incrementándose así la vida comercial del producto.

Una de las limitaciones del uso de las arcillas como nano-rellenos es la incompatibilidad entre la hidrofilia de la arcilla y la hidrofobicidad del polímero, lo cual puede dar lugar a la aglomeración de la arcilla en la matriz polimérica [10,11]. Una apropiada modificación orgánica de la arcilla es la clave para una exitosa exfoliación en la matriz polimérica, reduciéndose la hidrofilia de la arcilla y mejorando la compatibilidad con la misma [12]. Finalmente, se obtiene un material nanocompuesto con una disposición de la arcilla en forma laminar con un grosor de las mismas de tamaño nanométrico, de ahí que reciban el nombre de nanoarcillas.

En el caso de la MMT, una de las modificaciones más usuales llevadas a cabo y de la cual se ha obtenido un buen resultado, es la modificación por intercambio iónico. El cambio de iones inorgánicos de la MMT con iones orgánicos de amonio, alcaliamonios, da lugar a unos silicatos laminares organomodificados más organizados que mejoran la compatibilidad de la MMT con polímeros orgánicos [7,13].

Debido al amplio espectro de aplicaciones que tienen estas arcillas organomodificadas, el consumidor cada vez va a estar más expuesto a ellas. Por ello, se hace necesaria una rigurosa evaluación de estos nuevos materiales que se incorporan al mercado, dilucidando qué posibles efectos tóxicos podría ocasionar en el ser humano y las consecuencias de los mismos. Hasta el momento, la intoxicación por arcillas minerales ha ocurrido con más frecuencia por vía inhalatoria [10,14]. Sin embargo, al estar presente estas arcillas en materiales de envasado, la vía de entrada más común en la actualidad es la oral, pudiendo presentarse migración de las mismas desde el material de envasado hasta el alimento. Tateo y Summa [15], han descrito que la ingestión de arcillas minerales es común a bajas dosis teniendo en cuenta la presencia de las mismas en los productos, no sólo alimenticios sino también farmacéuticos y hierbas medicinales.

La evaluación toxicológica de estos productos puede llevarse a cabo tanto *in vitro* con *in vivo*. En la literatura científica podemos encontrar diversos estudios realizados *in vitro* con arcillas modificadas y los efectos observados tras la exposición a las mismas. Sharma y col. [16], evaluaron el potencial genotóxico de la MMT sin modificar, C®Na^+, así como el de la arcilla organomodificada Cloisite®30B (C®30B) en la línea celular intestinal humana Caco-2, concluyendo que la genotoxicidad observada podía deberse a la sal de amonio cuaternario usada como modificador de la arcilla. Por otro lado, también se ha evaluado la citotoxidad producida por exposición de las células hepáticas HepG2 a C®Na^+ y la arcilla modificada Cloisite®93A (C®93A), en las cuales se pudo observar un significativo descenso de la viabilidad celular [17]. En nuestro laboratorio, también se ha evaluado el potencial tóxico de arcillas modificadas en las líneas celulares mencionadas, observándose resultados diversos. Se sugiere por tanto que los modificadores empleados pueden estar involucrados en el perfil de toxicidad observado [18-21].

Por otro lado, los datos de ensayos *in vivo* realizados hasta el momento con este tipo de materiales aún son escasos, y, la información disponible no es representativa para la población humana, puesto que suelen ser ensayos a dosis repetidas en un periodo corto de tiempo. Por todo ello, el objetivo de este trabajo es determinar la toxicidad oral subcrónica de una arcilla modificada desarrollada por el Instituto Tecnológico del Embalaje, Transporte y Logística (ITENE) destinada a ser incorporada a ácido poliláctico para ser utilizada en la industria de envasado, así como del extracto de migración derivado del material nanocompuesto resultante, empleando como modelo experimental la rata Wistar. Se evaluó, en bazo, el contenido proteico, la posible peroxidación lipídica (LPO), la actividad de dos enzimas antioxidantes, superóxido dismutasa (SOD) y catalasa (CAT), y un análisis de contenido de metales (Al, Fe, Mg, Si y Ca) que hayan podido acumularse debido a la ingestión de la arcilla.

Materiales y Métodos

Materiales y reactivos

Los reactivos de uso general fueron proporcionados por Sigma Aldrich (Madrid, España) y VWR International Eurolab (España).

La arcilla organomodificada designada como Clay1 fue obtenida mediante una reacción de intercambio catiónico a partir de C®Na+ (Southern Clay Products, INC.), añadiendo una sal de amonio cuaternario, bromuro de hexadeciltrimetilamonio (HDTA), en una proporción seis veces mayor a la capacidad de intercambio catiónico (CEC) de la arcilla madre, de acuerdo con el método descrito por Mittal [22], Jordá- Beneyto y col. [23] y Jordá y col. [7]. La arcilla fue caracterizada mediante espectroscopía de infrarrojos (FTIR), análisis termogravimétrico (TGA) y difracción de rayos X (XRD) por Jordá-Beneyto y col. [24].

El extracto de migración se obtuvo de acuerdo a lo descrito en Maisanaba y col. [25] siguiendo la norma UNE-EN 1186-9:2002 [26] y utilizando como simulante alimentario el agua, ya que es el menos tóxico de entre los autorizados por la legislación vigente. Posteriormente, este agua fue utilizada como agua de bebida para el ensayo *in vivo*.

Animales y diseño experimental

Se realizó un estudio de toxicidad oral durante 90 días en ratas. Se usaron 30 ratas Wistar macho, con un peso medio de 240±2, proporcionadas por Janvier S.A.S (Francia). Las ratas fueron tratadas durante la semana de aclimatación con una alimentación estándar de laboratorio (Harlan 2014, Harlan Laboratories, Barcelona, España) y agua, ambos ad libitum, en una habitación con control de temperatura (23±1°C), con períodos de 12h de luz y 12h de oscuridad y libres de cualquier fuente de contaminación química. Posteriormente, los animales se dividieron en tres grupos, el grupo control (n=10), grupo expuesto a Clay1 (n=10) y grupo expuesto al extracto de Clay1 (n=10). El grupo control fue alimentado con dieta estándar y agua, al grupo expuesto a Clay1 se le administró una dosis de 40 mg/kg/día de la arcilla en la comida y, como bebida agua, y al grupo expuesto al extracto de Clay1 se le expuso a la dieta estándar y al extracto de migración como bebida. La dosis de arcilla fue seleccionada teniendo en cuenta el peor escenario de exposición para el ser humano. El consumo de comida y agua fue *ad libitum* en los tres grupos, registrándose éstos semanalmente, así como el incremento de peso de los animales.

Los animales fueron manipulados teniendo en cuenta las directrices establecidas para la protección de los animales utilizados en experimentación y otros fines científicos [27]. Así mismo, todos los procedimientos fueron aceptados previamente por el Comité Ético de la Universidad de Sevilla.

Extracción del bazo y preparación del sobrenadante postmitocondrial

Transcurridos los 90 días de exposición, las ratas se sometieron a un ayuno de 18h antes del sacrificio. El estudio se realizó en bazo por ser éste un órgano muy proclive a la acumulación de partículas [28] y por tanto podría ser una diana para este tipo de compuestos. Los órganos fueron lavados en frío con solución salina y posteriormente se pesaron. Las muestras se mantuvieron congeladas a -80°C hasta el análisis de los diferentes parámetros. Para el estudio de los biomarcadores de estrés oxidativo, el tejido se homogenizó usando un tampón compuesto por KH2PO4 50mM, Na4EDTA2H2O 1mM y albúmina de suero bovino (BSA). Realizada la homogenización se procedió a la centrifugación de las muestras (2500 x g durante 20min), eliminación del sobrenadante y recogida del homogenizado limpio en alícuotas de 2 mL.

Contenido proteico y LPO

El contenido proteico de las muestras de bazo fue determinado mediante el protocolo descrito por Bradford [29], usando como estándar la γ-globulina. Los resultados fueron expresados como mg proteína/g de tejido.

La LPO se cuantificó mediante el método del ácido tiobarbitúrico (TBA) [30]. Los valores fueron representados como nmoles de TBARS/g de tejido.

Enzimas antioxidantes

La actividad total de la SOD (EC 1.15.1.1) se determinó por el método de McCord y Fridovich [31]. Este ensayo depende de la capacidad de la SOD para inhibir la reducción del citocromo C mediada por el O_2 generado por el sistema de la xantina oxidasa. La actividad de la enzima fue medida a espectrofotométricamente a 505 nm. Los valores fueron representados como nkat/mg de proteínas. La actividad CAT (EC 1.11.1.6) fue calculada mediante el método de Beers y Sizer [32]. La reducción del peróxido de hidrógeno se mide espectrofotométricamente a 240 nm, usando cubetas de cuarzo de 1 mL con paso de luz de 1cm. Los resultados fueron expresados como nkat/mg de proteínas.

Análisis de metales

La presencia de Al, Fe y Mg en bazo fue determinada mediante espectrometría de masas con plasma de acoplamiento inductivo (ICP-MS) (Agilent 7500C, Agilent ICP-MS Systems, USA) utilizando los siguientes isótopos: Mg-24, Al-27 y Fe-56 (con celda de reacción octupolar presurizada con hidrógeno para eliminar la interferencia del ArO sobre el Fe-56). En el caso del Si y Ca, ambos fueron determinados mediante espectrometría de emisión atómica de plasma acoplado inductivamente (ICP-AES, Horiba Jobin Yvon, modelo última 2) empleando líneas de emisión 251.611 nm y 317.933 nm para Si y Ca, respectivamente. Estas determinaciones se llevaron a cabo en el Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS) en base a protocolos normalizados de trabajo realizando previamente una digestión del tejido con HNO_3 al 65%. Los datos se expresan como mg de metal/g de tejido.

Análisis estadístico

Los resultados se muestran como la media± DE de diez animales por grupo. El análisis estadístico llevado a cabo fue el análisis de la varianza (ANOVA) usando el software GraphPad InStat (GraphPad Software Inc., La Jolla, USA).

Resultados

Durante el periodo experimental no se produjo la muerte de ningún animal, siendo la evolución de los pesos y consumos normal. De igual forma, no se observaron efectos clínicos significativos.

Efectos sobre contenido proteico y LPO

El contenido proteico de los tres grupos experimentales, control, Clay1 y extracto de Clay1, fue muy similar (Fig. 1). La concentración proteica media resultó ser 32,5±3 mg de proteína/ g de tejido, no presentándose diferencias estadísticamente significativas entre los grupos.

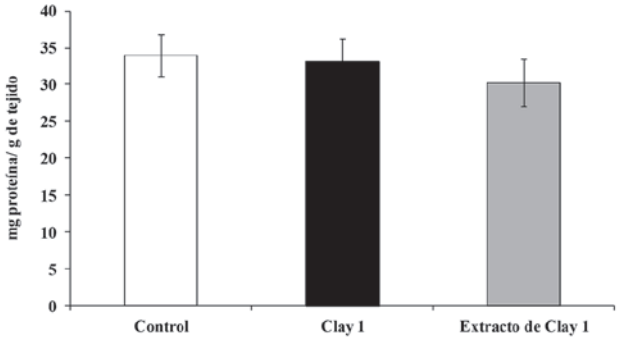

Figura 1. Contenido proteico (mg proteínas/mg tejido) de bazo de ratas control, ratas expuestas a 40 mg Clay1/kg rata/día y ratas expuestas a extracto de migración de Clay1 durante 90 días. Los valores están representados como la media± DE (n=10).

Los resultados obtenidos en LPO reflejaron que no hubo cambios significativos en ninguno de los grupos de exposición, Clay1 y extracto de Clay1, con respecto al grupo control (Fig. 2). En el grupo control fue obtenido un valor medio de 720±172 nmoles de TBARS/g de tejido. En el caso de los grupos tratados Clay1 y extracto de Clay1 fueron 712±164 y 676±69 nmoles de TBARS/g de tejido, respectivamente.

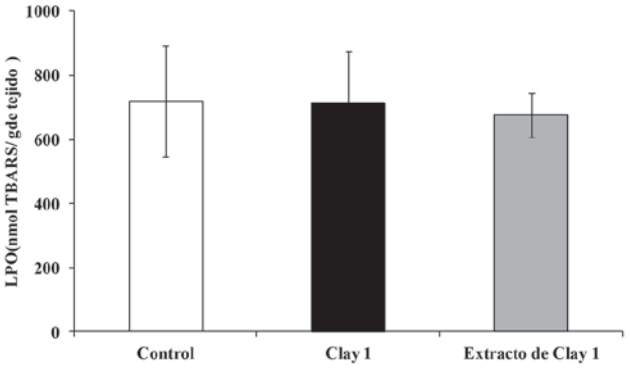

Figura 2. Valores de LPO en bazo de ratas control, ratas expuestas a 40 mg Clay1/kg rata/día y ratas expuestas al extracto de migración de Clay1 durante 90 días. Los valores están representados como la media± DE (n=10).

Efectos sobre enzimas antioxidantes

La actividad SOD no se alteró tras la exposición a 40 mg/kg/día de Clay1 tras el periodo de exposición, no observándose diferencias significativas con respecto el grupo control (Fig. 3a). Del mismo modo, los valores obtenidos en el caso del grupo expuesto al extracto de Clay1 fueron similares a los obtenidos en el grupo control. Los valores medios por grupo fueron: 133±26, 141±35 y 122±15 nkat/mg

de proteínas para el grupo control, Clay1 y extracto de Clay1, respectivamente.

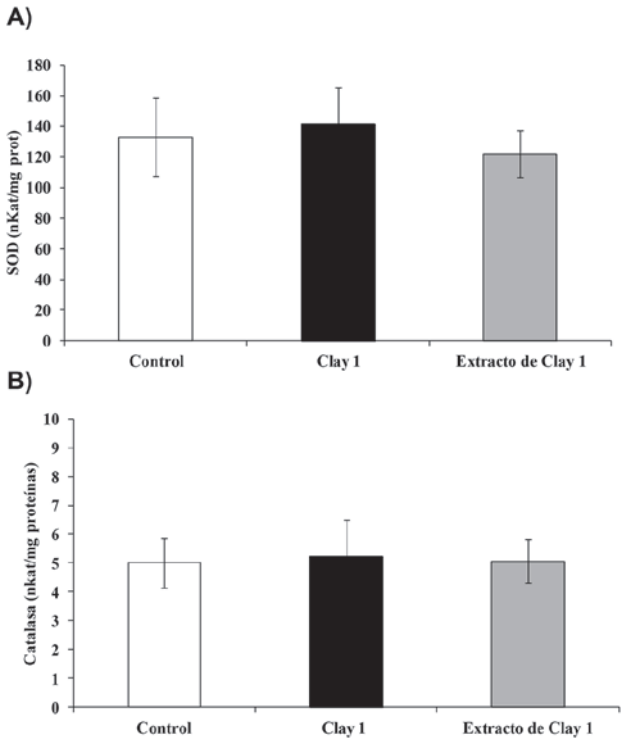

Figura 3. Actividad enzimática de SOD (Fig.3A) y CAT (Fig.3B) (nkat/mg proteína) en bazo de ratas control, ratas expuestas a 40 mg Clay1/kg rata/día y ratas expuestas al extracto de migración de Clay1 durante 90 días. Los valores están representados como la media± DE (n=10).

En el caso de CAT, se obtuvieron resultados similares en los tres grupos (Fig. 3B), no afectando a la actividad de la enzima la exposición a la arcilla modificada o al extracto de migración. Las actividades medias de la enzima en los grupos experimentales, control, Clay1 y extracto de Clay1, fueron 5±0,8, 5,2±1,2 y 5±0,7 nkat/mg de proteínas, respectivamente.

Determinación de metales en bazo

En la tabla 1 se recogen los datos obtenidos en el análisis del contenido de metales en el bazo. En ningún caso se observaron cambios significativos tras 90 días de exposición.

Tabla 1. *Contenido de Al, Ca, Fe, Mg y Si (mg metal/g tejido) en bazo de ratas control, ratas expuestas a 40 mg Clay1/kg rata/día y ratas expuestas a extracto de migración de Clay1 durante 90 días. Los valores están representados como la media± DE (n=10).*

Grupos Experimentales	Media±DE (mg/g)				
	Al	Ca	Fe	Mg	Si
Control	0,215±0,089	0,198±0,089	1,536±0,230	0,153±0,012	0,011±0,004
Clay 1	0,138±0,057	0,132±0,087	1,640±0,699	0,153±0,011	0,009±0,007
Extracto de Clay 1	0,170±0,078	0,147±0,049	1,787±0,327	0,133±0,019	0,008±0,005

Discusión

El uso de arcillas minerales en diferentes aplicaciones industriales, y más concretamente en la industria alimentaria, está en auge debido a los buenos resultados técnicos obtenidos. Por tanto, la exposición a la mismas se ve aumentada pudiéndose ver comprometida la seguridad del ser humano. La arcilla modificada Clay1 ha sido desarrollada con el fin de reforzar envases alimentarios, y es considerada por tanto como material de contacto alimentario [24]. La exposición del consumidor a compuestos químicos presentes en el material de envasado de alimentos podría ocurrir debido a la migración de los mismos desde el envase [33]. Tanto la migración como la toxicidad de una determinada sustancia se convierten en los dos factores principales que podrían definir el riesgo para el consumidor expuesto a estos nuevos materiales incorporados a los envases [34]. En relación a su toxicidad, se han realizado ensayos *in vitro* con Clay1 en la línea celular intestinal humana Caco-2 y en la línea hepática HepG2 [18,19]. Los resultados obtenidos mostraron una muy baja toxicidad en el rango de concentraciones ensayadas (0-8 µg/mL). Únicamente en uno de los biomarcadores (contenido proteico total) y en la línea celular Caco-2 tras 48h de exposición a la concentración más alta se obtuvieron diferencias significativas. Concentraciones superiores no pudieron ensayarse debido a la hidrofobicidad de la arcilla.

Según la Comisión Europea [35], para que una sustancia que va a entrar en contacto con alimentos se comercialice, ésta debe pasar antes por una serie de evaluaciones, entre la que se encuentra una evaluación toxicológica precisa que recoge ensayos de diversa índole. Entre estos ensayos debe realizarse un estudio de toxicidad oral de 90 días. En el presente estudio, los resultados obtenidos, teniendo en cuenta que se ha trabajado con el peor escenario de exposición (migración total de la arcilla), no han mostrado efectos tóxicos notables en las ratas. Otros autores también han llevado a cabo estudios *in vivo* por vía oral con arcillas, aunque usando periodos de exposición más cortos. Li y col. [36] llevaron a cabo un ensayo de toxicidad oral en ratas, a las cuales se les expuso por una única vez a 4 dosis diferentes de una arcilla proveniente de MMT, sin encontrar finalmente efectos adversos tras 14 días desde la administración. Por otro lado, resultados similares fueron obtenidos por Baek y col. [37] después de realizar un ensayo de toxicidad oral aguda en ratones expuestos a MMT. No obstante, considerando la aplicación de estas arcillas en el envasado de alimentos el escenario de exposición más realista se correspondería con una ingesta de dosis bajas y repetidas en el tiempo como el empleado en el presente estudio.

Los resultados observados en el presente estudio, son consecuentes con los obtenidos previamente por nuestro equipo de investigación tras realizar en los mismos grupos experimentales un análisis de la bioquímica clínica del suero, interleukina-6 (IL-6) como marcador inflamatorio y contenido en glutatión reducido y oxidado (GSH/GSSG). En ningún caso se observaron diferencias significativas con respecto al grupo control en las determinaciones llevadas a cabo [38]. De forma similar en hígado y riñón tampoco se encontraron cambios en los tres biomarcadores considerados [39,40]. Esta ausencia de efectos adversos contrasta con los resultados obtenidos con ensayos *in vitro* con la MMT y/o arcillas organomodificadas derivadas de ella por otros autores [18-21,25]. En los mismos cabe destacar la generación de especies reactivas de oxígeno (ERO) y por tanto producción de estrés oxidativo por exposición de dichas arcillas a las diferentes líneas celulares. Sin embargo, los estudios anteriores fueron llevados a cabo con arcillas diferentes a Clay1, pudiendo intervenir en el perfil de toxicidad de la arcilla el modificador empleado.

Meibian y col. [41] expusieron la línea celular humana de linfoblastos B (HMy2, CIR), a otras arcillas diferentes a la MMT, dos tipos de bentonitas (BP), activa y no activa, además de partículas de cuarzo y

yeso. Estos autores evaluaron *in vitro* la actividad de SOD y la producción de ácido malonildialdehído (MDA) por exposición a estos materiales, observando una inhibición de la enzima antioxidante tras la exposición de las bentonitas y no tras la exposición a las otras partículas, e incluso presentándose una mayor inhibición por exposición de la BP activa. En el caso de la producción de MDA ocurre algo similar a lo anterior, produciéndose de forma muy significativa un aumento de la LPO en la línea celular tras la exposición de ambas bentonitas. En este caso, la modificación de la bentonita activa no estaría involucrada en el perfil de toxicidad presentado.

En cuanto a la presencia de metales en órganos como consecuencia de la exposición a arcillas, varios autores han realizado diferentes estudios. Mascolo y col. [42] llevaron a cabo un ensayo *in vivo* de toxicidad oral aguda centrado en la ingestión y distribución de elementos químicos presentes en distintas arcillas, ya que el organismo puede absorber dichos elementos procedentes de las mismas. Estos autores expusieron a los animales a una dosis de 450 mg/kg de arcilla, sacrificándolos a los tres días. No se observaron efectos tóxicos notables en las ratas aunque sí un incremento de las concentraciones de los elementos traza en determinados órganos. Finalmente los autores concluyeron que la exposición a arcillas podría derivar en serios problemas de salud por el incremento en el contenido metálico observado en los órganos.

Nuestro estudio llevado a cabo en bazo de ratas Wistar expuestas a 40 mg/kg/día de Clay1 y a su extracto de migración durante 90 días no dio lugar a ninguna alteración en los parámetros estudiados, ni a un incremento del contenido metálico en los grupos experimentales, pudiendo concluir que la arcilla modificada podría no comprometer la seguridad del consumidor en un futuro si ésta se comercializara.

Agradecimientos

Los autores desean agradecer al Ministerio de Ciencia e Innovación (AGL2010-21210) y a la Junta de Andalucía (AGR5969) por la financiación de este estudio y la concesión de una beca predoctoral asociada a este último. También, agradecer al Servicio de Radioisótopos y Microanálisis del CITIUS por el análisis de metales en bazo.

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CAPÍTULO 14/ CHAPTER 14

RESULTADOS PENDIENTES DE PUBLICACIÓN COMO ARTÍCULOS / *RESULTS PENDING PUBLICATION*

14.1. Development, Characterization and Cytotoxicity of Novel Silanes Modified Clays intended to Packaging

Introduction

Nanocomposites materials based on modified clays imbued in polymers as reinforcements, have been intensively studied in the last years aiming to obtain final products with enhanced mechanical, thermal and barriers properties for the food industry (Smolander and Chaundry, 2010). Among the layered silicates most used in this research area, montmorillonite (Mt), commercially known as Cloisite®Na^+ (CNa^+), stand out by its high aspect ratio, capacity for swelling and ion exchange, softness, high solvent resistance, good mechanical strength, high capacity for delamination and low cost (Betega de Paiva et al., 2008). The clay presents a 2:1 structure, composed by two tetrahedral layers formed by Si and O atoms, fused with an octahedral layer with aluminum and magnesium atoms bonded to oxygen and hydroxyl groups (Jordá-Beneyto et al., 2014).

The Mt has 1-nanometer-thick layers when is incorporated to a polymer, stacked on each other and loosely bound by Van der Waals forces, which allow the penetration of water and/or other polar molecules. To compensate for the negative charges generated by the isomorphic substitutions which occur in the tetrahedral and octahedral sheets of the Mt, exchangeable cations such as Na^+, $Ca2^+$ and Li^+ are electrostatically fixed in the interlayer space of the clay minerals (Bertuoli et al., 2014; Paul and Robenson, 2008). However, a simple dispersion of a Mt in a polymer matrix will not produce a nanocomposite with better properties compared to the bulk material. This is due to the poor interfacial interactions between Mt hydrophilic reaction sites and the highly hydrophobic polymers chains (Pisticelli et al., 2010). The solution for the aforementioned disadvantages can be achieved by introducing organic groups; providing more supports to the interaction between clays and polymers (Silva et al., 2011).

The most useful chemical modifications procedure to prepare a more organophilic Mt is a cation exchange (Ray and Okamoto, 2003; Paul and Robenson, 2008: Pavlidou and Papaspyrides, 2008). One of the most commonly performed modifications is by quaternary ammonium salts, producing modified organoclays with an hydrophobic character and a greater interlayer space (Betega de Paiva et al., 2008; Jordá-Beneyto et al., 2014). However, due to the quaternary ammonium salt degradation

at high temperatures through Hoffman-degradation, the thermal instability of the organoclay becomes a strong limitation in the processing of clay mineral/ polymer nanocomposites (Xie et al., 2001, 2002; Wang et al., 2012).

For these reasons, the grafting silylation reaction of hydrophobic groups into the layer surfaces has attracted great attention. It is usually performed through the reaction between silane coupling agents and the reactive silanol groups which are located at the broken edges of the clay platelets and at the structural defects located at the interlayer and external surface (Herrera et al., 2005; Di Gianni et al., 2008; Pisticelli et al., 2010). In summary, the irreversible grafting reaction is the solution to obtain chemically modified clay in which the molecule of the silanes modifiers is bonded to the layers of the selected clay, in this case Mt (Wypych, 2004). Several authors have investigated the modification of clays with silanes incorporated to different polymers, taking in account different modification methods and obtaining various results, improving or declining the properties compared to the unmodified clay and the bulk polymers (He et al., 2005; Shen et al., 2007; Di Gianni et al., 2008; Pisticelli et al., 2010; Silva et al., 2011).

Respect to the toxicity of silanes modified clays used in packaging the information is very limited, even non-existent, being necessary a strict evaluation according to the authorities in charge of the regulation about new materials intended as food contact materials (EFSA, 2011, 2015).

For all this, the present work studies the modification of Mt with different silanes. The modifiers used were 3-aminopropyltriethoxysilane (APTES), obtaining Clay3, and vinyltrimethoxysilane (VMTS) in two different proportions (4 and 8% w/w), giving Clay4A and Clay4B, respectively. The development of the organomodified clays was carried out with aqua and N_2 atmosphere as dispersion media and functionalization conditions. X-ray diffraction (XRD), thermogravimetric analysis (TGA) and Fourier transform infrared (FTIR) spectroscopy were employed to characterize the modified clay minerals. Moreover, a toxicological study with the modified clays was also performed, considering a cytotoxicity biomarker in human cell lines from intestinal and hepatic origin.

Materials and Methods

Supplies and Materials

Purified sodium montmorillonite (Mt or CNa^+) was purchased from Southern Clay Products, with moisture content between 4 and 9 %. The cation exchange capacity (CEC) of MMT is 92.6 mequiv/100g. Silanes were supplied by Sigma Aldrich (Madrid, Spain). APTES and VMTS with ≥98 % of purity were used for the preparation of the modified clays, Clay3, Clay4A and Clay4B, respectively.

Culture medium, fetal bovine serum and cell culture reagents were obtained from Gibco (Biomol, Spain). Chemicals for the cytotoxicity assay were provided by Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

Organomodified clays preparation

The silylation of the Mt was performed according to Silva et al. (2011) with modifications. Briefly, 10g of dry MMT were dispersed in 500mL of distilled water at 80°C during at least 2 hours with the help of a UltraTurrax (IKA, Spain) working between 9000- 11000 rpm. Then, 5.7 mL of APTES or VMTS for Clay3 and Clay4A, and a higher amount, approximately 10 mL, of VTMS for Clay4B were added. The resulting dispersion was stirred for 30 min under nitrogen atmosphere. The dispersion was left overnight at room temperature and the reaction product was washed with water at 60°C and recovered by centrifugation at 3500 rpm. Then, the silane modified clays was dried between 60-70°C during 24h. After, the dry clays were crushed and maintained other 24h in the oven under vacuum. A grafting reaction between the clay and the silanes is carried out in an aqueous solution.

Materials characterization methods

Clays were characterized using different techniques. FTIR (Fourier Transform InfraRed) Spectra were obtained on a Tensor 27spectrometer (Bruker): For this purpose an ATR (Golden Gate Single Reflection Diamond ATR, Specac) objective was used. This technique was used to characterize clays. For each sample 64 scans were recorded with a resolution of 4 cm-1.WAXD (wide-angle X-ray diffraction) analysis were performed on clay powders (clay pellets were obtained by pressing), using a Bruker AXS D5005 difractometer equipped with a Cu hollow cathode (l=0.154 nm) and a oscillation detector. The X-ray diffraction pattern was measured from 3 to 30 degrees (2θ) at a scan rate of 0.02 ºC/min. Thermogravimetric analysis (TGA) was performed on

clay powders using a TA Q5000IR thermobalance. 10.0 mg of sample was heated from 25 to 900 °C at a heating rate of 20 °C/minute in nitrogen atmosphere.

Model systems for the cytotoxicity test

Caco-2 cell line derives from a human colon carcinoma (ATCC® HTB-37, passages 10-18) and HepG2 derives from a human hepatocellular carcinoma (ATCC®HB-8065, passages 5-15) both of them were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 cells were cultured in monolayer in Eagle´s medium (EMEM) supplemented with 10% of FBS and 100 U/mL penicillin and 100Ug/mL streptomycin (Gibco, Biomol, Spain). Caco-2 cell line was maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 µg/mL gentamicine, 2 mM L-glutamine, and 1 mM pyruvate. HepG2 cell line was cultured in monolayer in EMEN supplemented with 10% of FBS, 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin/ streptomycin (Gibco, Biomol, Spain). Cells were grown near confluence in 75-cm^2 plastic flasks at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer hemocytometer and viability was determined by the Trypan Blue exclusion test. Both cell lines were plated at a density of 7×10^5 cells/mL to perform the experiments.

Clay test solutions

The test concentrations of the three clays were determined individually in previous experiments to avoid interferences with the method of measurement. For this reason the absorbance of clay solutions (1000 µg ml–1 and serial half dilutions) were measured at 0, 24 and 48 h. The highest concentrations selected were the ones that did not show statistical differences versus the control (culture medium without clays). Stock solutions were 1000 µg ml–1 for the three clays. An ultrasonic tip (Dr. Hielscher, Germany) at amplitude of 40% for a total time of 30 s carried out in 10 s sequential steps was employed to disperse the test concentrations. From the initial solution, serial dilutions in free-serum medium were prepared (1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 µg/ mL of Clay3, Clay4A and Clay4B). After replacing the previous medium, the exposure solutions were added to systems, and incubated at 37 °C for 24 and 48h.

Cytotoxicity assay

From the initial solution, serial dilutions in free-serum medium of the three modified clays were prepared (0-250 µg/mL). After replacing the previous medium, the exposure solutions were added to systems, and incubated at 37 °C for 24 and 48h. After that time, the basal cytotoxicity was analyzed by the Protein Content (PC). The use of this biomarker has been recognized by the European Union Reference Laboratory for alternatives to animal testing for basal cytotoxicity studies. PC is a very useful endpoint to assess cytotoxicity, as it gives data about cell damage with independence of the toxic mechanism involved (Pichardo et al., 2007). PC was quantified in situ, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, to determine the total cell number present in the culture. The culture medium was replaced by 200 µl NaOH 0.1 M and after 2 h incubation at 37 °C, 180 µl was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm (Infinite M200, Tecan).

Calculations and Statitical Analysis

Results are expressed in graphs as mean ± standard deviation (s.d.). Statistical analysis was carried out using analysis of variance (ANOVA), followed by a Dunnet´s test. Differences were considered significant from $p < 0.05$; $p < 0.01$.

Results

Organo-modified clays characterization

Fourier Transform InfraRed (FTIR)

Three different modified clays were obtained from Mt following the method described previously. The presence of modifiers in the clay was determined by FTIR. Figs. 1a, 1b and 1c, gave strong evidence that the experimental procedure used in this work was successful in altering the chemistry of Mt. The ATR spectra in Figure 1 show the presence of a peak under 1200 cm−1, which corresponds to Si-O absorptions and OH bands that are typical of clay minerals and also marked the presence of the silanes modifiers. Inner hydroxyl groups, lying between the tetrahedral and octahedral sheets, give the absorption as being near 3620 cm−1, as can be seen in all the spectra in Fig. 1

(Madejova, 2003).Two strong bands near 2930 and 3620 cm−1 are present in spectra of the modified clays .

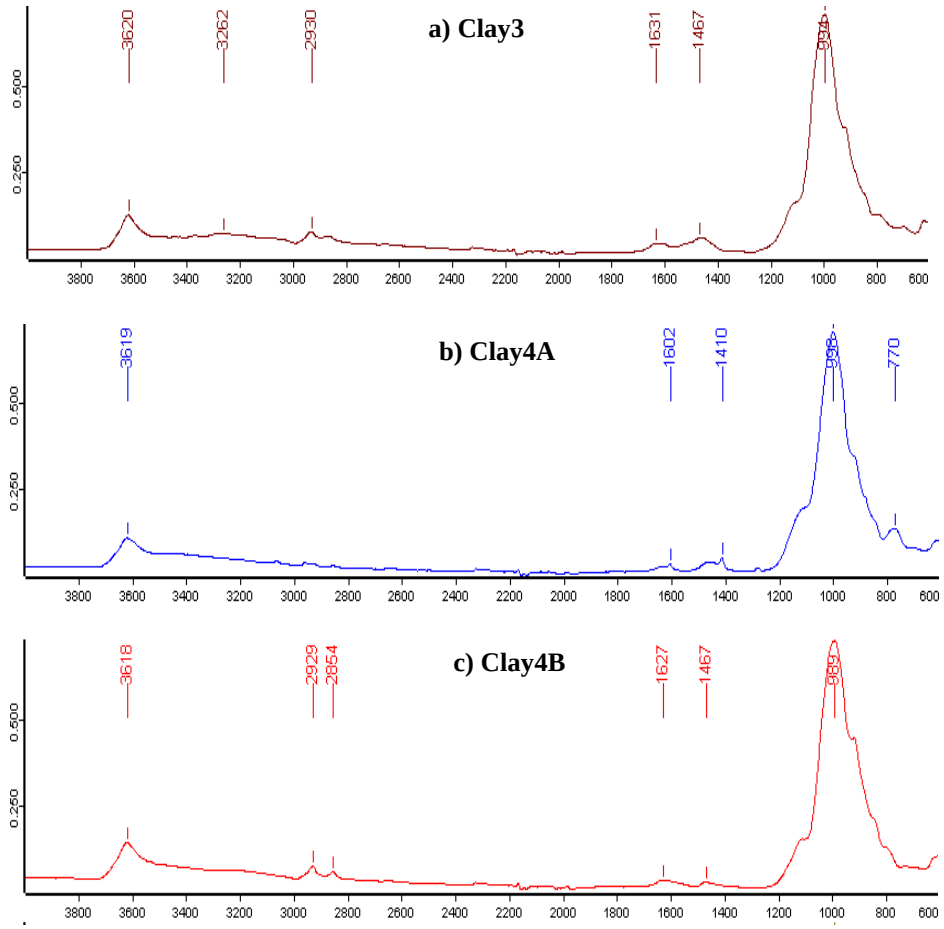

Figure 1. Fourier transform infrared spectrum of a) Clay3, b) Clay4A and c) Clay4B

Wide-angle X-ray diffraction

X-ray diffraction of the raw clay and modified ones are shown in Fig. 2. Interlayer space has been especially enlarged with the silylation in Clay 3. Results of interlayer space are shown in **¡Error! No se encuentra el origen de la referencia.**. It can be observed that the interlayer space in Clay3 is twice of the raw clay (Mt) and higher than in Clay4A and B modified clays. The measured d (001) spacing of pristine Mt is 9.99 Å (2θ = 8.85). After cation exchange with silanes, the d (001) spacing became 18.80 Å (2θ = 4.70) in the case of Clay3, 12.33 Å (2θ = 7.17) in the case of Clay4A and 12.13 Å (2θ = 7.29) in the case of Clay4B. These results provide evidence that the modifier

intercalates inside the Mt galleries. This is an important step to reach a good exfoliation in the final nanocomposite.

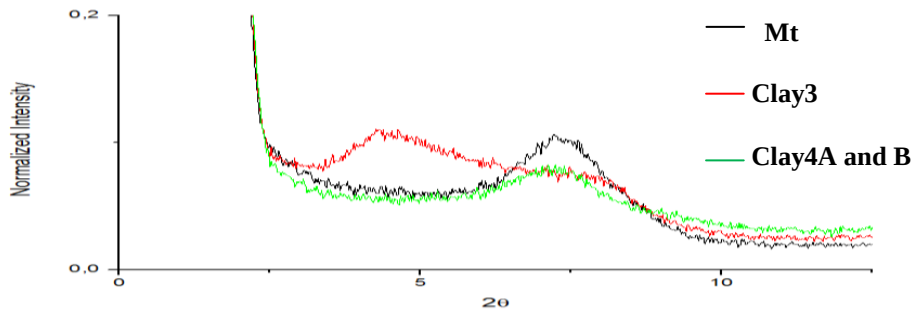

Figure 2. X-ray spectrum of raw clay N116, and organosilane-modified clays (Clay3, Clay4A and B).

Samples	2θ	d_{001} (Å)
Mt	8.85	9.99
Clay3	4.70	18.80
Clay4A	7.17	12.33
Clay4B	7.29	12.13

Table 1. Interlayer space results of raw clay Mt, and organosilane-modified clays (Clay3, Clay4A and Clay4B).

Thermogravimetric analysis (TGA)

The exchange degree of the modifier inside the Mt was determined by TGA. The results are shown in Figs 3a, 3b. Xie et al. (2001) determined that the TGA curves of organo-modified layer silicates are divided into four parts: (1) the free water region in the temperature range below 200°C; (2) the region where organic substances evolve in the temperature range 200–500°C; (3) the structural water region in the temperature range 500–800°C; and (4) a region between 800 and 1000°C where organic carbon reacts in some yet unknown way. The weight losses (%) of organic substances for Clay3, Clay4A and Clay4B, between 200°C and 500°C approximately, were: 16.2%, 14.6% and 11.2%, respectively. A higher loss in the weight of sample Clay3, was observed.

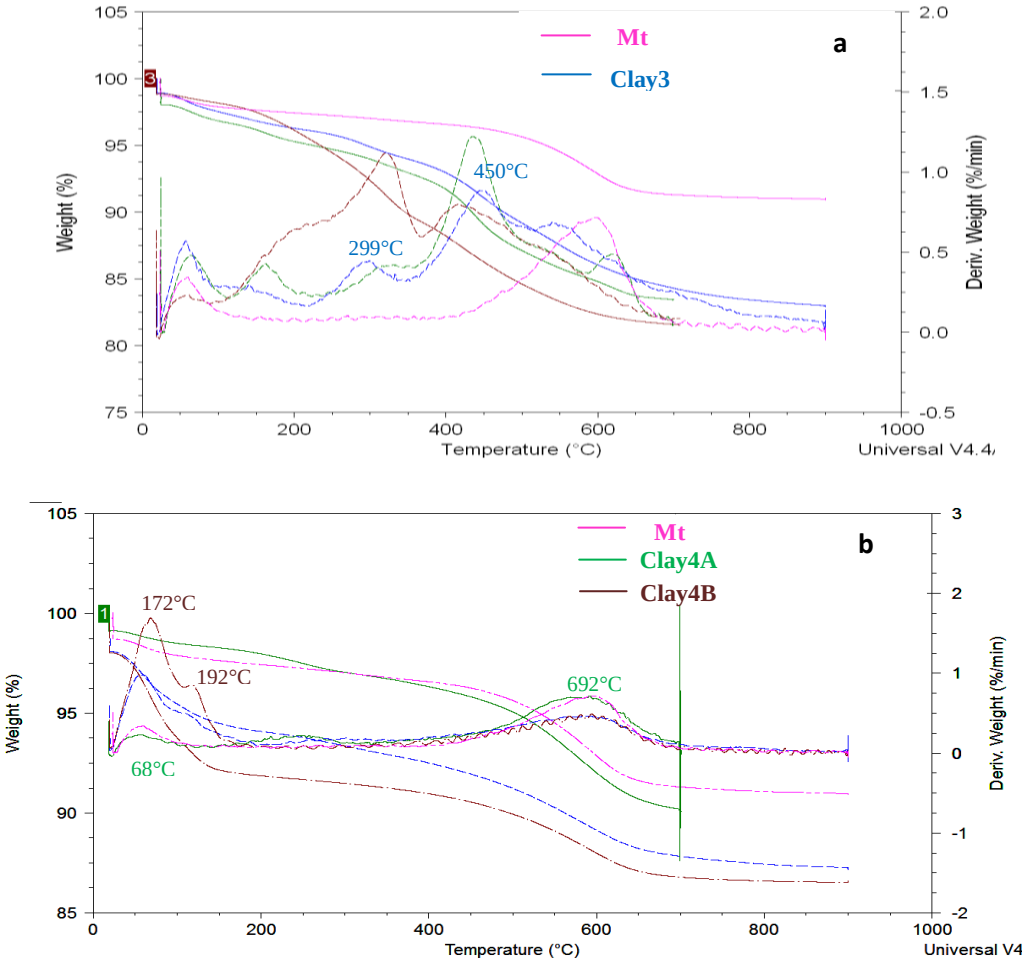

Figure 3. Thermogravimetric analysis (TGA) of the raw and modified clay: a) Clay3 and b) Clay4A and Clay4B

Cytotoxicity tests

Caco-2 and HepG2 cells exposed to Clay3 remained unaltered with respect to the control cells after both times of exposure at all concentrations tested (Fig. 4a, d). However, Clay4A and Clay4B produced a decrease in cell viability in both cell lines after 24 and 48h of exposure, being stronger with Clay4B (Fig. 4b, c, e, f).

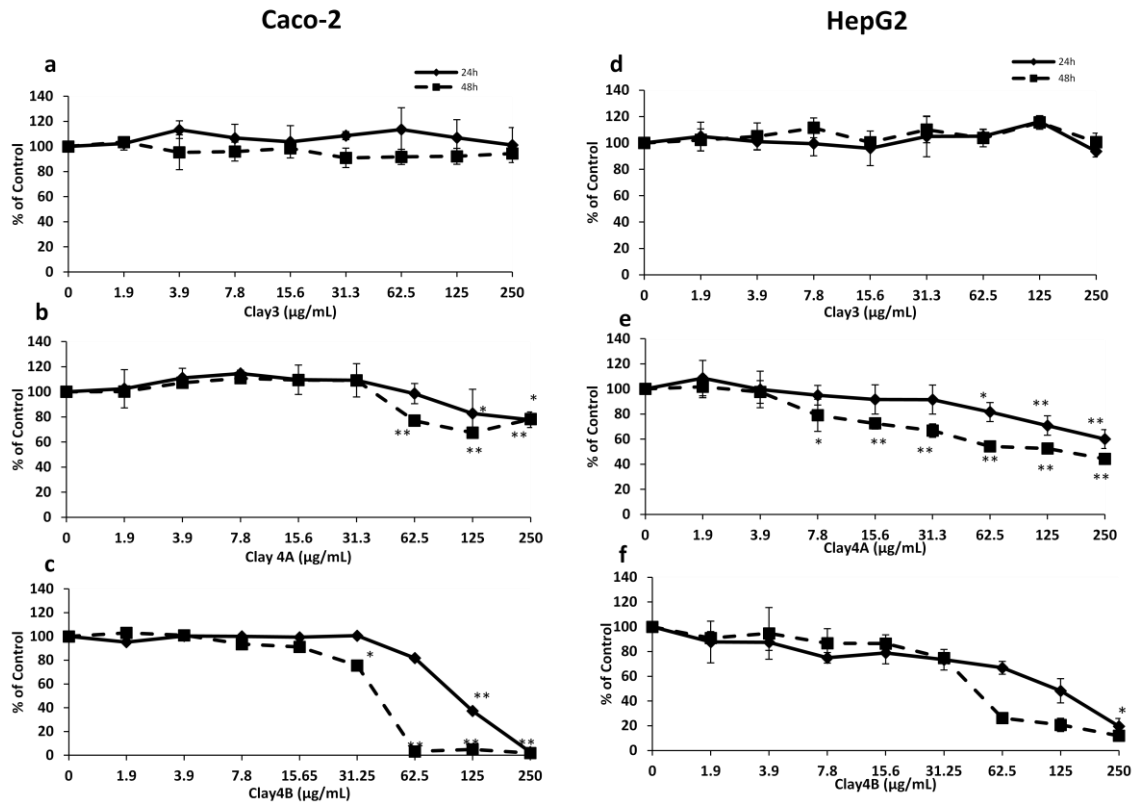

Figure 4. Total protein content of Caco-2 (a, b, c) and HepG2 (d, e, f) cells after 24 h and 48 h of exposure to 0–250 µg/mL Clay3 (a, d), Clay4A (b, e) and Clay4B (c, f). Results from 3 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences were considered significant from $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Discussion

The development and characterization of three silane-modified clays and their cytotoxicity effects have been performed in the present work. Others authors have described the incorporation and characterization of similar modifiers in other layered silicates, obtaining results which agree with exposed results in this study (Mansur et al., 2002; Ahmed et al., 2009). Regarding cytotoxic effects, to extent out knowledge, no toxicity information is available in the bibliography. In chapter 13.2, 13.3 and 13.4 are showed the results obtained with other cytotoxicity biomarkers, neutral red uptake (NR) and MTS reduction, in the same cell lines selected in this work. Similarly, no cytotoxic effects were obtained with Clay3, however, a significant decrease in cell viability with Clay4´s modified clays was observed. Moreover, the results of genotoxicity and stress oxidative results of the modified clays were also carried out, giving an absence of toxicity in Clay3 and positive response with the other ones.

Several authors reported the effects of other organomodified clays which have quaternary ammonium salts as modifiers (Sharma et al., 2010; Lordan et al., 2011; Houtman et al., 2014). Different results were obtained with each modified clay, as well as, contradictory or inconclusive data depending on the conditions applied. Taking into account the results obtained an individually case by case evaluation of clays under different conditions are required in order to reach the most complete toxicity information.

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14.2. *TOXICITY EVALUATION OF A NEW SILANE-MODIFIED CLAY AND ITS MIGRATION EXTRACT FROM A NANOCOMPOSITE INTENDED TO FOOD PACKAGING*.

Introduction

In order to improve the features of food packaging, the food industry is developing new materials based on the incorporation of organomodified clays into polymers. Clays or layered silicates typically have a stacked arrangement of silicate layers (nanoplatelets) with nanometric thickness, hence they are named as nanoclays. The resulting material should present better physical properties than the polymer without clay, enhancing therefore the perdurability of packaged food.

The Technological Institute of Packaging, Transport and Logistic (ITENE, Valencia) has developed a novel silane organoclays using aminopropyltriethoxysilane (APTES), obtaining Clay3. The complete process is described in the chapter 13.1.

Considering that the this nanoclay is intended to be used in food packaging and consumer could be in contact with them, toxicological assessment is required. In the present study the toxicity of Clay3 was evaluate in the human cells lines Caco-2 and HepG2 using basal cytotoxicity biomarkers. In addition, the potential mutagenicity was assayed using 5 *Salmonella typhimurium* strains. In addition, the migration of the major metals presented in clays structure was measured in food stimulants obtained from polypropylene (PP) and Clay3.

Materials and Methods

Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

Clays and clays test solutions

Clay3 has been developed and characterized as it was described in chapter 13.1. The test concentrations were selected taking into account previous dispersion experiments in order to avoid interferences with the measurement system. The highest

concentration tested was 250µg/mL. Test solutions were prepared in serum-free medium. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

Cell cultures

Caco-2 cell line (human colon carcinoma, ATCC® HTB-37) and HepG2 (human hepatocellular carcinoma epithelial cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 1 mM pyruvate. In the case of Caco-2 50 µg/mL gentamicine was added and 100 U/mL penicillin/ streptomycin (Gibco, New Zealand) was used for HepG2. Cells were grown near confluence in 75-cm2 plastic flasks at 37°C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer hemocytometer and viability was determined by the Trypan Blue exclusion test. Cells were plated at a density of 7.5×10^5 cells/mL to perform all experiments.

Cytotoxicity assays

From the initial solution, serial dilutions in medium were prepared (0–250 µg/mL). Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions in culture medium without serum were added to the systems, and incubated at 37°C for 24 and 48h. The basal cytotoxicity endpoints were supravital dye neutral red celular uptake (NRU) and tetrazolium salt reduction (MTS).

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Furthemore, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the

procedure of Baltrop et al. (1991). Both assays were performed according to Maisanaba et al. (2013).

Ames test

The incorporation version of the Ames test has been described briefly in Maisanaba et al. (2015) and was performed according to the recommendations of Maron and Ames (1983) and following the principles of OCDE guideline 471 (1997).

Migration assay

The presence of characteristic metals of the clays structure in polypropylene (PP)–Clay3 and only PP (as control) unfiltered extracts was analyzed. Al, Fe and Mg were quantified by (ICP-MS) (Agilent 7500C, Agilent ICP-MS Systems, USA). These determinations were carried out in the Scientific- Techniques Services (University of Oviedo) based on standard operating protocols.

Statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $p < 0.05$.

Results

Cytotoxixity assays

No significant differences was observed in Caco-2 cells exposed up to 250 µg/mL Clay3 in NRU endpoint after 24h or 48h of exposure (Fig. 1a). Similarly, no remarkable cytotoxic effect was observed in MTS reduction assay, except for a significant decrease at the highest concentration and exposure time assayed (250 µg/mL after 48h) (Fig 1b).

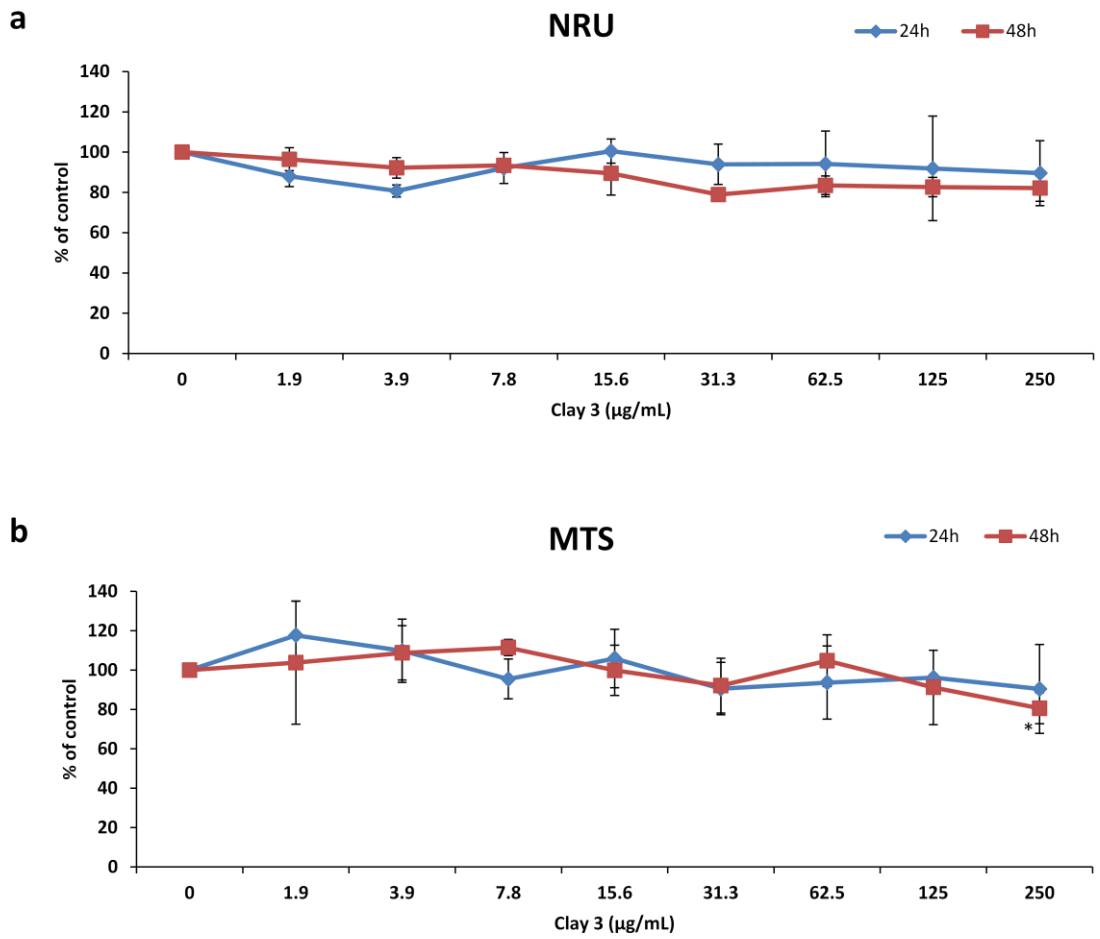

Figure 1. Neutral red uptake (a) and MTS reduction (b) of Caco-2 cells after 24 h and 48 h of exposure to 0–250 µg/mL Clay3. Results from 3 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences were considered significant from $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Similar pattern was observed when HepG2 cells were exposed to Clay 3 for 24 and 48 h. No significant change could be distinguished in any assay performed (Fig. 2). No significant differences was observed in Caco-2 cells exposed up to 250 µg/mL Clay3 in NRU endpoint after 24h or 48h of exposure (Fig. 1a). Similarly, no remarkable cytotoxic effect was observed in MTS reduction assay, except for a significant decrease at the highest concentration and exposure time assayed (250 µg/mL after 48h) (Fig 1b)..

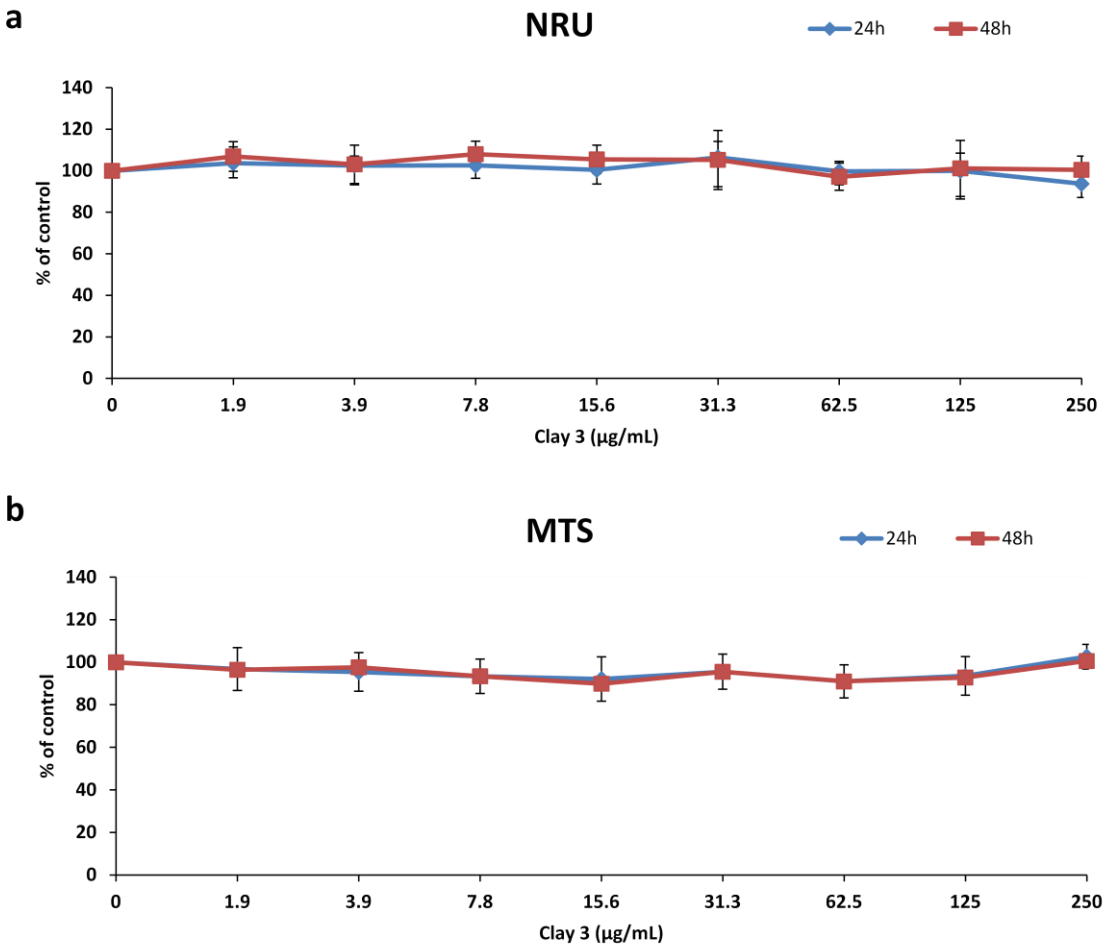

Figure 2. Neutral red uptake (a) and MTS reduction (b) of HepG2 cells after 24 h and 48 h of exposure to 0–250 µg/mL Clay3. Results from 3 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences were considered significant from $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Ames test

After exposure to Clay3, no significant changes were detected in any of the strains exposed to the organomodified clay in absence or presence of S9 fraction (Table 1).

	Concentration (µg/mL)	TA97A		TA98		TA100		TA102		TA104	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Clay 3	Negative controls	270±45	250±37	29±9	28±7	119±33	114±26	292±66	272±23	295±17	358±31
	15.63	238±50	312±37	19±5	53±1	117±22	89±21	273±34	268±45	287±37	327±55
	31.25	269±26	309±49	20±7	47±1	95±13	73±11	300±50	241±22	245±26	269±62
	62.5	276±39	280±56	21±7	46±5	97±21	109±28	309±54	358±17	400±45	282±51
	125	273±51	250±37	24±6	37±11	106±5	101±29	289±46	282±57	258±30	298±57
	250	284±34	324±6	23±5	47±3	95±18	93±9	257±42	219±29	214±35	272±21
	Positive controls	680±71**	617±25**	>1000**	586±49**	362±6**	322±22**	591±13**	661±29**	638±28**	752±6**
	DMSO	328±15	310±46	17±3	20±4	120±7	131±7	228±21	236±30	258±22	212±15

Table 1. Results of Ames test conducted with Clay3 for three independent experiments. MilliQ water was used as negative control and DMSO as solvent for positive controls. Data are given as mean ± SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/TA98/TA102/TA104 without S9 mix: 2-NF (0.1 µg/plate) and TA100 without S9 mix: NaN3 (1 µg/plate). Positive controls for all strains with S9: 2-AF (20 µg/plate). * Significantly different from control ($P \leq 0.05$). ** Very significantly different from control ($P \leq 0.01$).

Migration assay

Some of the metals contained in the film PP-Clay3 were detected in the simulant. In the case of ethanol 10%, three metals released from the film to the migration media: Mg and Si (with quantities significantly different from the control group) and Ca (being its enhancement very significant in comparison to the control group). In the case of isooctane, only Mg increased significantly with the rest of the metals showing similar values to the control group.

	Al	Ca	Fe	Mg	Si
Ethanol 10% extract					
Control	25±7.8	110.5±19.1	0.4±0.3	7.3±2.5	12500.7±561.1
Clay3	3.3±1.2	376.5±2.1**	0.2±0.1	48.7±7.1*	14693±243.5*
Isooctane extract					
Control	60.3±5.5	275.7±65.6	42±7.1	20±4	4270.7±321.9
Clay3	41.3±2.9	376±17	34.7±15.2	54.3±8.1*	3648.3±809.5

Table 2. Al, Ca, Fe, Mg and Si content in the migration extracts with etanol 10% or isooctane from PP and PP–Clay3 (all the concentrations are expressed in µg/L). All values are expressed as mean ± SD, n=3. *Significant ($p \leq 0.05$), **very significant ($p \leq 0.01$) and ***extremely significant($p \leq 0.001$) differences from control.

Discussion

Clay3 have shown no cytotoxic effects except for a significant decrease observed at 250 µg/mL after 48 h of exposure in Caco-2 cells when MTS assay was performed. Similarly, no remarkable effect was observed at any concentration and exposure time for HepG-2. In addition, no potencial mutagenicity was discernible in Ames test for any bacterial strain, both with and without metabolic activation.

In the migration assay, several metals migrate from the film containing Clay3 to the food simulant, in the case of ethanol 10% Ca, Mg and Si and in isooctane only Mg was detected. However, no cytotoxic effect were recorded for the extract of migration exposed to Caco-2 and HepG2 (data not shown).

Considering all the results obtained in the present work, Clay3 seems to be safe. Therefore, it could be used in the manufacturing of films for its use in food packaging.

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14.3. Toxicological assessment of two silane-modified clays in human hepatoma cells and *Salmonella typhimurium* strains

Introduction

Currently, the incorporation of organomodified clays into polymers intended for packaging industry is a great alternative in order to enhance the products perdurability. Clays or layered silicates typically have a stacked arrangement of silicate layers (nanoplatelets) with nanometric thickness, hence they are named as nanoclays. The development of nanocomposites is a new strategy to improve physical properties of polymers, including mechanical strength, thermal stability, and gas barrier properties (Arora and Padua, 2010). The incorporation of nano platelets of clays into polymers has been used to create a tortuous path that impedes the passage of water, oxygen, aroma and tainting compounds, hence reducing the rate of diffusion. Often, incorporation of only a low mass fraction such as a few percent in the nanocomposites, can increase barrier properties many-fold compared to the polymer alone (Smolander and Chaundry, 2010; Bradley et al., 2011)

Montmorillonite (Mt), one of the most used clays, is a layered silicate belonging to the structural family of the 2:1 phyllosilicates. The presence of inorganic cations on the planar surface of Mt layers makes them hydrophilic and hence ineffective in hydrophobic polymers. Cation exchange reactions is one of the possibilities to resolve this problem, replacing these inorganic cations with organic cationic surfactants which intercalate into the clay gallery, producing organically modified Mt with increased interlayer spacing, hydrophobic surface and improved interactions with organic polymers (De Azeredo, 2013). However, these kind of modifiers are degradated at high temperatures, being a disadvantage in some aspects. In this manner, the grafting silylation reaction in a real alternative which solve the above mentioned handicap, being irreversible and giving also to the polymers the improvements in the technological aspects (Chapter 13.1; Pisticelli et al., 2010). In this case, the modifiers used are silanes. There are a lot of studies which described the properties of these new silanes organomodified clays (Di Gianni et al., 2008; Pisticelli et al., 2010; Silva et al., 2011), but the toxicity information about them is scarce or non-existent.

The Technological Institute of Packaging, Transport and Logistic (ITENE, Valencia) has developed two novel silane organoclays using vinyltrimethoxysilane

(VMTS), obtaining Clay4A and Clay4B, both of them based also Mt but with different proportions of silane, 4 and 8%, respectively as it is described in the chapter 13.1..

Due to the increase in the manufacture of nanoclay-containing products, data on the toxicological effects of these substances is required. In view of the limited toxicological information of these novel clays, in this study we aim to evaluate the toxicity of Clay4A and Clay4B in the human hepatocellular cell line HepG2 and *Salmonella typhimurium* strains. For this purpose, basal cytotoxicity biomarkers and mechanistic biomarkers of oxidative stress, genotoxicity and mutagenicity have been investigated.

Materials and Methods

Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

Clays and clays test solutions

Clay4A and Clay4B have been developed and characterized as it was described in chapter 13.1. The test concentrations for both clays were selected taking into account previous dispersion experiments in order to avoid interferences with the measurement system. The highest concentrations tested were 250 µg/mL for both. Test solutions were prepared in serum-free medium. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

Cell culture

HepG2 (human hepatocellular carcinoma epithelial cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% of FBS (Gibco, New Zealand), 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin/ streptomycin (Gibco, New Zealand). Cells were grown at 37°C and 5% CO_2 in humidified atmosphere. Cells were used at passages between 8 and 18.

Cytotoxicity assays

From the initial solution, serial dilutions in medium were prepared (0–250 µg/mL for Clay4A and Clay4B). Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions in culture medium without serum were added to the systems, and incubated at 37°C for 24 and 48 h. The basal cytotoxicity endpoints were supravital dye neutral red celular uptake (NRU), and tetrazolium salt reduction (MTS).

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Furthemore, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). Both assays were performed according to Maisanaba et al. (2013).

Only when cytotoxicity was observed, mechanistic biomarkers were determined. In this case, the mean effective concentration (EC50) of the most sensitive cytotoxicity endpoint was chosen as the higher exposure concentration to investigate mechanistic biomarkers along with the fractions EC50/2 and EC50/4. In this sense, the EC50, EC50/2 and EC50/4 for Clay4A were 213, 106.5 and 53.25 µg/mL and for Clay4B were 85, 42.5 and 21.25 µg/mL, respectively.

Glutathion content and Reactive Oxygen Species generation, Comet Assay and Ames test

The production of reactive oxygen species (ROS) was assessed in 96 wells microplates using the dichlorofluorescein (DCF) assay according Puerto et al. (2010). Cellular glutathione (GSH) content was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl) as described by Jos et al. (2009).

Regarding to the comet assay, it aims to detect DNA strand breaks. The comet assay was performed following the steps previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011).

The incorporation version of the Ames test has been described briefly in Maisanaba et al. (2015) and was performed according to the recommendations of Maron and Ames (1983) and following the principles of OCDE guideline 471 (1997).

Statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $p < 0.05$. EC50 values, mean effective concentration, concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls, were determined by linear interpolation.

Results

Cytotoxixity assays

HepG2 cells exposed to Clay4A showed significant differences in NRU endpoint from 125 µg/mL after 24h of exposure, and from 1.9 µg/mL after 48h. However, MTS biomarker did not display any change at the conditions tested after Clay4A exposure (Fig. 1a, 1c). On the other hand, the results obtained with Clay4B were more drastic than with the other clay. Hepatoma cells experimented a significant viability decrease with Clay4B in NRU endpoint from 31.25 µg/mL after 24 and 15.6 µg/mL 48h of exposure, being more sensitive than MTS, which showed significant differences from 62.5 µg/mL after both times of exposure (Fig. 1b, 1d).

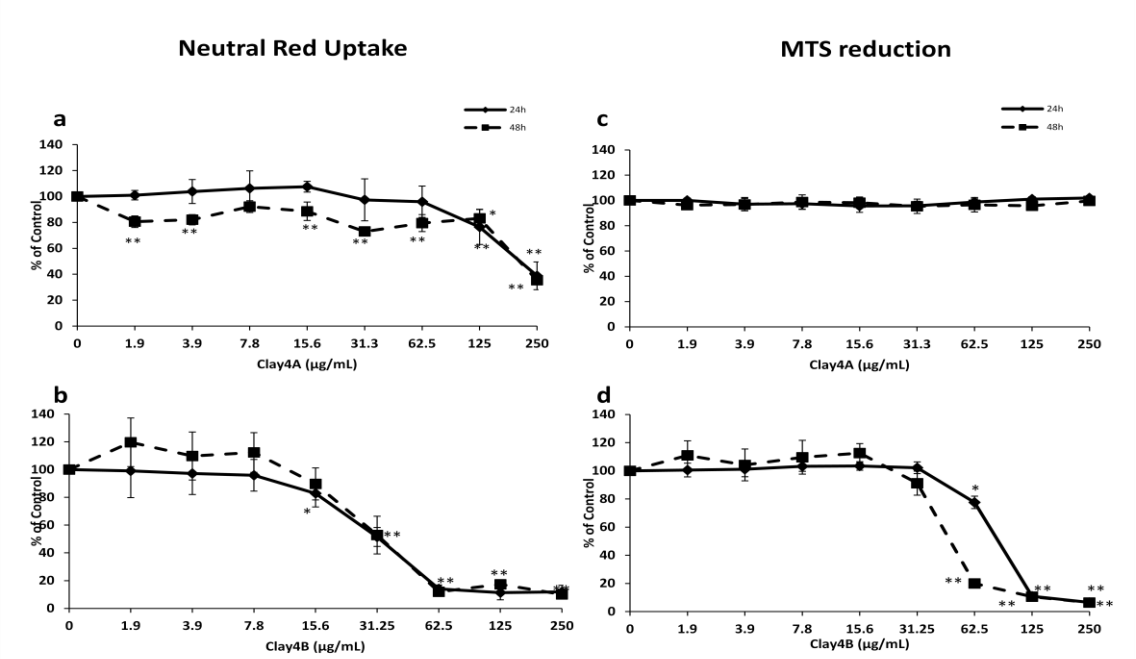

Figure 1. Neutral red uptake (a, b) and MTS reduction (c, d) of HepG2 cells after 24 h and 48 h of exposure to 0–250 µg/mL Clay4A (a, c) and Clay4B (b, d). Results from 3 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences were considered significant from $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

GSH content and ROS production

When HepG2 cells were exposed to Clay4A during 24 and 48h, no significant alteration on GSH content was observed at any of the exposure concentrations in comparison to the control group (Fig. 2a). In contrast, a concentration-dependent increase of ROS production was observed at the concentrations assayed after both times of exposure (Fig. 2c). On the other hand, hepatoma cells exposed to Clay4B showed significant decreases of GSH content at the two highest concentrations tested after 24 and 48h of exposure (Fig.2b), as well as, an increase of ROS production in all cases (Fig. 2d).

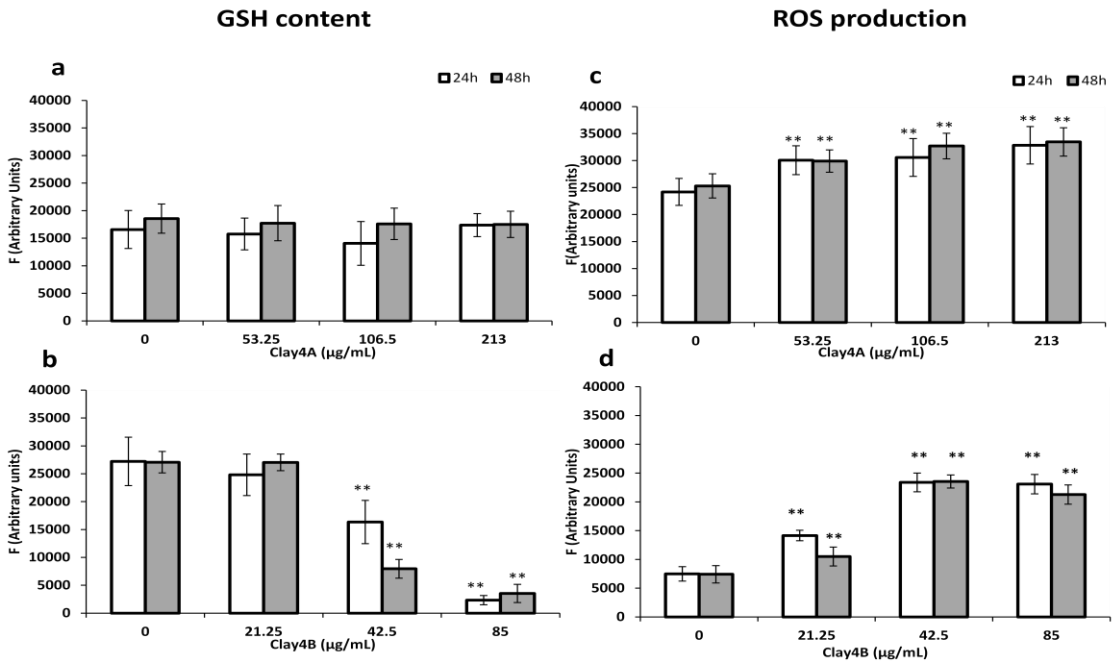

Figure 2. GSH content (a, b) and ROS production (c, d) in HepG2 cells after 24 and 48 h of exposure to 53.25, 106.5 or 213 µg/mL Clay4A (a, c) or, 21.25, 42.5 or 85 µg/mL Clay4B (b, d). Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d. **significantly different from control ($p \leq 0.01$).

Comet assay

Clay4A did not induce DNA strand-breaks in Hep-G2 cells at the concentrations tested after 24 and 48 h of exposure (Fig. 3a). In regard to HepG2 cells exposed to 21.25 and 43 µg/mL of Clay4B, no significant variations of the DNA strand breaks were detected after 24 h and 48 h of exposure. However, the highest exposure concentration (85 µg/mL) led to a significant ($p < 0.05$) increase of DNA in the tails, compared to that of the control (Fig. 3b).

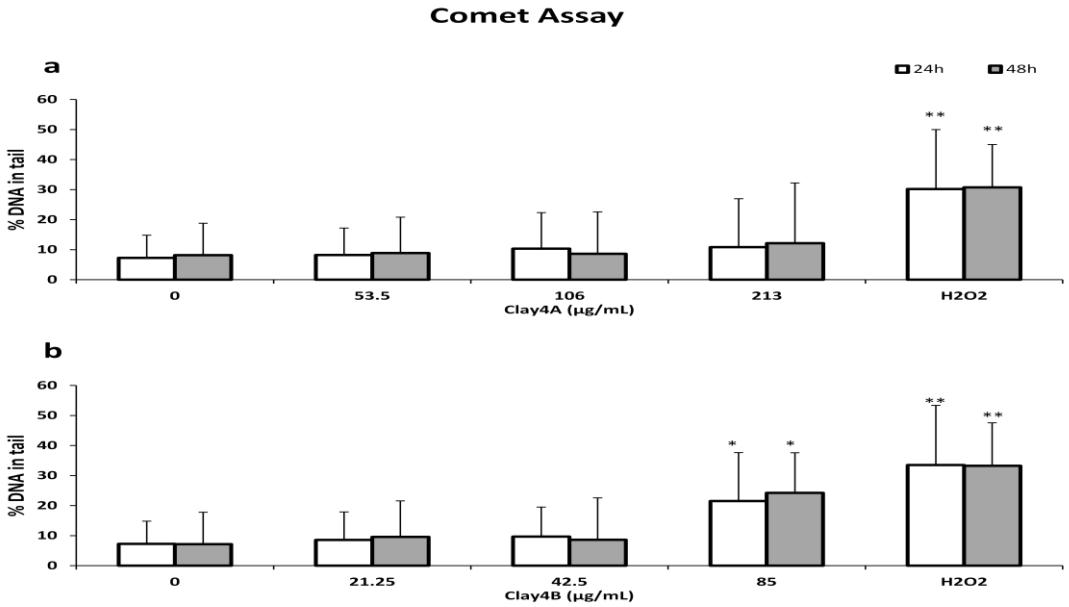

Figure 3. Comet assay results of HepG2 cells after 24 and 48 h of exposure to 53.25, 106.5 or 213 µg/mL Clay4A (a) or, 21.25, 42.5 or 85 µg/mL Clay4B (b). Results from 3 independent experiments with 2 replicates/experiment. All values are expressed as mean ± s.d. *significantly different from control ($p \leq 0.05$) or ** very significantly different from control ($p \leq 0.01$).

Ames test

After exposure to Clay4A, no significant changes were detected in any of the strains exposed to the organomodified clay in absence or presence of S9 fraction (Table 1). Clay4B showed significant increases in the revertant colonies compared to the negative control in TA102 from 62.5µg/mL without and with S9, while no change was recorded after the exposure in the other strains (Table 1).

	Concentration (µg/mL)	TA97A		TA98		TA100		TA102		TA104	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Clay4A	0	350±66	239±39	29±8	37±2	166±50	141±44	202±49	205±22	268±44	265±43
	15.65	378±60	267±54	23±6	43±1	89±19	82±9	260±66	282±79	287±66	273±66
	31.25	322±50	303±51	28±14	41±10	98±10	101±13	205±12	240±56	267±52	435±31
	62.5	250±38	236±59	18±4	30±4	94±21	91±25	166±21	203±56	234±63	275±32
	125	269±38	309±16	31±14	24±6	94±28	99±31	228±67	252±63	279±32	331±89
	250	254±70	328±49	26±6	27±3	90±18	95±31	250±5	262±49	300±79	267±76
	+ controls	546±27••	557±27••	>1000••	423±21••	384±18••	331±26••	537±52••	571±13••	518±11••	594±65••
	DMSO	209±66	184±38	25±2	30±6	115±5	113±17	250±65	231±75	328±66	431±42
Clay4B	0	152±31	152±21	23±5	17±8	105±24	112±26	123±41	150±39	240±27	259±46
	15.65	130±13	130±36	30±11	24±5	137±27	134±36	161±38	250±36	250±75	236±25
	31.25	150±11	153±28	24±6	21±8	103±29	97±25	205±46	266±58	207±31	255±57
	62.5	206±11	153±28	24±7	23±7	110±21	75±10	221±69*	319±58*	193±27	244±29
	125	205±53	251±65	20±3	22±5	126±34	113±21	292±56*	347±65*	326±34	361±66
	250	224±85	252±45	21±9	23±5	149±27	136±30	272±80*	307±21*	246±60	237±34
	+ controls	676±78••	676±10••	>1000••	>1000••	458±37••	395±4••	741±24••	774±11••	734±29••	741±46••
	DMSO	297±37	320±34	27±7	39±2	119±16	115±3	267±13	381±32	373±25	360±13

Table 1. Results of Ames test conducted with Clay4A and Clay4B for three independent experiments. MilliQ water was used as negative control and DMSO as solvent for positive controls. Data are given as mean ± SD revertants/plate for three replicates for each concentration in each experiment. Positive controls: TA97A/TA98/TA102/TA104 without S9 mix: 2-NF (0.1 µg/plate) and TA100 without S9 mix: NaN3 (1 µg/plate). Positive controls for all strains with S9: 2-AF (20 µg/plate). * Significantly different from control ($P \leq 0.05$). ** Very significantly different from control ($P \leq 0.01$).

Discussion

Both clays have presented a pronounced cytotoxicity and ROS production, however, only Clay4B showed pronounced genotoxic and mutagenic effects. This could be due to the silane amount in their structures, indicating a proportional response when the vyniltrimethoxysilane quantity is higher. Similar results were obtained in Caco-2 cells exposed to Clay4A and Clay4B (chapter 13.4), where drastic cytotoxic effects were observed with the organomodified clay with higher silane amount, as well as, genotoxic effects by the comet assay. Nevertheless, it is important to note the significant ROS production observed in adenocarcinoma cells exposed to Clay4A but not to Clay4B.

To the extent our knowledge, toxicity data about these clays are not available in the scientific literature till the moment. Other authors reported some studies about the non modified raw clay (Cloisite®Na^+ (CNa^+)) and other kinds of organomodified clays with quaternary ammonium salts in the same cell line. In this sense, Lordan et al. (2011) described a decrease in the viability of HepG2 cells exposed to CNa^+ and Cloisite®93ª, a modified clay. However, only CNa^+ induced intracellular ROS formation which coincided with increased cell membrane damage, whilst ROS generation did not play a role in Cloisite®93A-induced cell death. On the other hand, CNa^+ did not induce DNA strand-breaks in HepG2 cells and Caco-2 cells after 24 h of exposure, as tested with the alkaline comet assay (Sharma et al., 2010; Maisanaba et al., 2014). However, Cloisite® 30B induced genotoxic effects in hepatoma cells and contradictory results were obtained in adenocarcinoma cells (Sharma et al., 2010; Maisanaba et al., 2013; Maisanaba et al., 2014).

Overall, the results obtained depend on the cell lines selected, concentrations and times assayed, as well as, the modifiers used and their quantity in the clay.

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14.4. Cyotoxicity, oxidative stress and genotoxicity assays of silanes-modified clays in the human intestinal cell line Caco-2.

Introduction

Mineral clays present a vast variety of applications, many of them derived from their properties (Sharma et al., 2010). Montmorillonite (Mt) is one of the main components of bentonite, a layered silicate widely used due to its good swelling capacity, high specific surface area, good cation-exchange capacity, high platelet aspect ratio, strong adsorptive power, high structural stability, chemical inertia, strong capacity to form stable suspensions, modificable surface, etc. (Hu y col., 2007; Mallakpour and Dinari, 2011; Baek y col., 2012). Layered clays are of great interest in food packaging industry, since they confer better barrier properties when incorporated to composite materials (Rhim and Ng, 2007). Nielsen (1967) explained this fact through the winding path around the clay platelets, forcing the permeable gas to travel a longer way to diffuse through the packaging. Polymers incorporated with nanoparticulated clays are one of the first nanocomposite polymers to be used in the food packaging industry. One limitation of clays is their incompatibility with hydrophobic polymers due to their hydrophilic properties (Elmore y Andersen, 2003; Zeng y col., 2005). In order to solve this inconvenient, the surface modification of these clays through cation exchange with organic cations is used, giving place to organomodified clays compatible with those polymers used in food packaging industry (Sharma y col., 2010; Maisanaba et al., 2014a). However, this modification is not stable at high temperatures, so the grafting silylation reaction became a real alternative since it is irreversible and also confers polymers technological improvements (Pisticelli et al., 2010). In this sense, the Technological Institute of Packaging, Transport and Logistics (ITENE, Valencia, Spain) has developed two Mt-based clays with their surface modified with different quantities of vinyltrimethoxysilane (VMTS): Clay4A (4% silane) and Clay4B (8% silane). Since these clays could migrate to food and also due to their novelty, a study concerning their safety must be carried out. As it can be assumed, the oral route is the main exposure route to humans. Tateo and Summa (2007) reported that the ingestion of clays is common at low doses, since they are present in food preparations, pharmaceutical preparations, and herbal remedies. Several authors have evaluated the potential toxicity of modified clay minerals destined to food packaging in target cell lines (Maisanaba et

al., 2015; Houtman et al., 2014; Maisanaba et al., 2014a,b; Maisanaba et al., 2013; Lordan et al., 2011; Sharma et al., 2010).

In the present study we aim to evaluate the toxicity of C Clay4A and Clay4B in the human intestinal cell line Caco-2. For this purpose, basal cytotoxicity, oxidative stress biomarkers, apoptosis and necrosis biomarkers, and DNA damage were investigated.

Materials and Methods

Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

Clays and clays test solutions

Clay4A and Clay4B have been developed and characterized as it was described in chapter 13.1. The test concentrations for both clays were selected taking into account previous dispersion experiments in order to avoid interferences with the measurement system. The highest concentrations tested were 250 µg/mL for both. Test solutions were prepared in serum-free medium. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

Cell culture

Caco-2 cell line derives from a human colon carcinoma (ATCC® HTB-37) and was obtained from the American Type Culture Collection. Caco-2 cell line was maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 µg/mL gentamicine, 2 mM L-glutamine, and 1 mM pyruvate. Cells were grown near confluence in 75-cm2 plastic flasks at 37°C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer hemocytometer and viability was determined by the Trypan Blue exclusion test. Cells were plated at a density of 7.5×10^5 cells/mL to perform all experiments.

Cytotoxicity assays

From the initial solution, serial dilutions in medium were prepared (0–250 µg/mL for Clay4A and Clay4B). Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions in culture medium without serum were added to the systems, and incubated at 37°C for 24 and 48 h. The basal cytotoxicity endpoints were supravital dye neutral red celular uptake (NRU), and tetrazolium salt reduction (MTS).

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). Both assays were performed according to Maisanaba et al. (2013).

Oxidative stress study

After replacing the previous medium, exposure solutions were added to cells and incubated at 37ºC for 24 and 48 h. The oxidative stress endpoints measured were reactive oxidative species (ROS) content and glutathione (GSH) levels.

The production of ROS was assessed in 96-well microplates using the dichlorofluorescein (DCF) assay. Cells were incubated with 20 µM DCFH-DA in culture medium at 37ºC for 30 min, and then washed with phosphate buffered saline (PBS) and resuspended in PBS. The formation of the fluorescent oxidised derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as fluorescent arbitrary units (Puerto et al., 2010).

GSH content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos et al., 2009). After the cell exposure, medium was discarded and cells were incubated at 37ºC for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the excitation/emission wavelengths of 380/460. Results were expressed as arbitrary units.

Comet Assay

The comet assay was performed to detect DNA strand breaks. Caco-2 cells were seeded on to 12-well tissue culture-treated plates (Corning Costar Corporation, New York, USA). Approximately $3.5x10^5$ cells in each well were exposed with different concentrations of Clay4A (0, 62.5, 125, 250 µg/mL) and Clay4B (0, 10, 20, 40 µg/mL) for 24 and 48 h. To monitor the ongoing process of the assay, a negative control (cells treated with medium without fetal calf serum) and a positive control (cells treated with a solution of 100 µM H_2O_2) were included. After treatments, cells were washed and detached in PBS. The comet assay was applied as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, cells were resuspended in PBS at a concentration of $2.5x10^6$ cells/mL. This suspension was mixed with 1% low melting point agarose and placed on a microscope slide. Once the gels had become solid, the slides were dipped into lysis solution at 4°C. All nucleotides were denatured in a high pH buffer (0.3 M NaOH, 1 mM EDTA, pH 13). Electrophoresis was carried out at approximately at 25 V (300 mA) and the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold nucleic acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20x objective) coupled via a CCD camera to an image analysis system (DP controller–DP manager). Images of randomly selected nuclei (≥ 100) per experimental point were analyzed with the image analysis software (Comet Assay IV; Perceptive Instruments, Suffolk, England, UK). The results from four independent experiments are expressed as percentage of tail DNA and are shown as box plots.

Flow Cytometry

The detection of apoptosis was performed following the method described by Cárdeno et al. (2013) with modifications. Briefly, Caco-2 cells ($7.5x10^5$ cells/mL) were seeded in 6-well plates. After 24 h of incubation, cells were treated in presence of different concentrations of Clay4A (62.5, 125 and 250 µg/mL) and Clay4B (10, 20 and 40 µg/mL). Moreover, a negative control without the presence of any compound, and a positive control with the presence of curcumin 40 µM were prepared. After 24 or 48 h of exposure, media were collected and cells were detached by trypsinization (0.05% Trypsin– EDTA) and collected in 0.5 mL of MEM without serum. Afterwards, cells

were centrifuged at 4500 rpm during 3 min at 4ºC, resuspended and washed with ice-cold PBS, centrifuged, and resuspended in ice-cold 1x binding buffer (BB) to $5x10^5$ cells/mL. Cells were incubated with 25 µL/mL Annexin V-FICT and 20 µg/mL propidium iodide (PI) solution (Annexin V-FICT Apoptosis Detection Kit, eBioscience, Vienna, Austria). Four different groups of cells were obtained based on their stainability: those unstainable with annexin V or PI [annexin(–)/PI(–)]: viable cells (quadrant E3); those stainable with annexin V but unstainable with PI [annexin(+)/PI(–)]: early apoptotic cells (quadrant E4); those stainable with both annexin V and PI [annexin(+)/PI(+)]: late apoptotic cells (quadrant E2); and those unstainable with annexin V but stainable with PI [annexin(–)/ PI(+)]: primary necrotic cells (quadrant E1). The untreated population was used to define the basal level of apoptotic and dead cells.

Analysis of metal content in the migration extracts

The presence of characteristic metals of the clays structure in polypropylene (PP)–Clay4 and only PP (as control) unfiltered extracts was analyzed. Al, Fe and Mg were quantified by (ICP-MS) (Agilent 7500C, Agilent ICP-MS Systems, USA). These determinations were carried out in the Scientific- Techniques Services (University of Oviedo) based on standard operating protocols.

Statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant from $p < 0.05$. EC50 values, mean effective concentration, concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls, were determined by linear interpolation.

Results

Cytotoxicity assays

NR uptake performed by Caco-2 cells exposed to Clay4A remained unaltered with respect to the control cells after 24, whereas at 48h experienced a significant ($p<0.05$)

decrease with respect to the control at 125 and 250 μg/mL, respectively (Fig.1a). In the case of MTS reduction, Caco-2 cell line showed no alterations at 24 h. Moreover, after 48h, MTS metabolization was significantly reduced between 62.5 and 250 μg/mLClay4A (Fig 1b).

On the other hand, the NR uptake with Clay4B presented a significant ($p<0.05$; $p<0.001$) decrease from 125 μg/mL after 24 h of exposure, and from 62.5 after 48h. (Fig. 1c). Similarly, MTS reduction presented a decrease from the concentration of 62.5 μg/mL with significant differences after both exposure times (Fig. 1d).

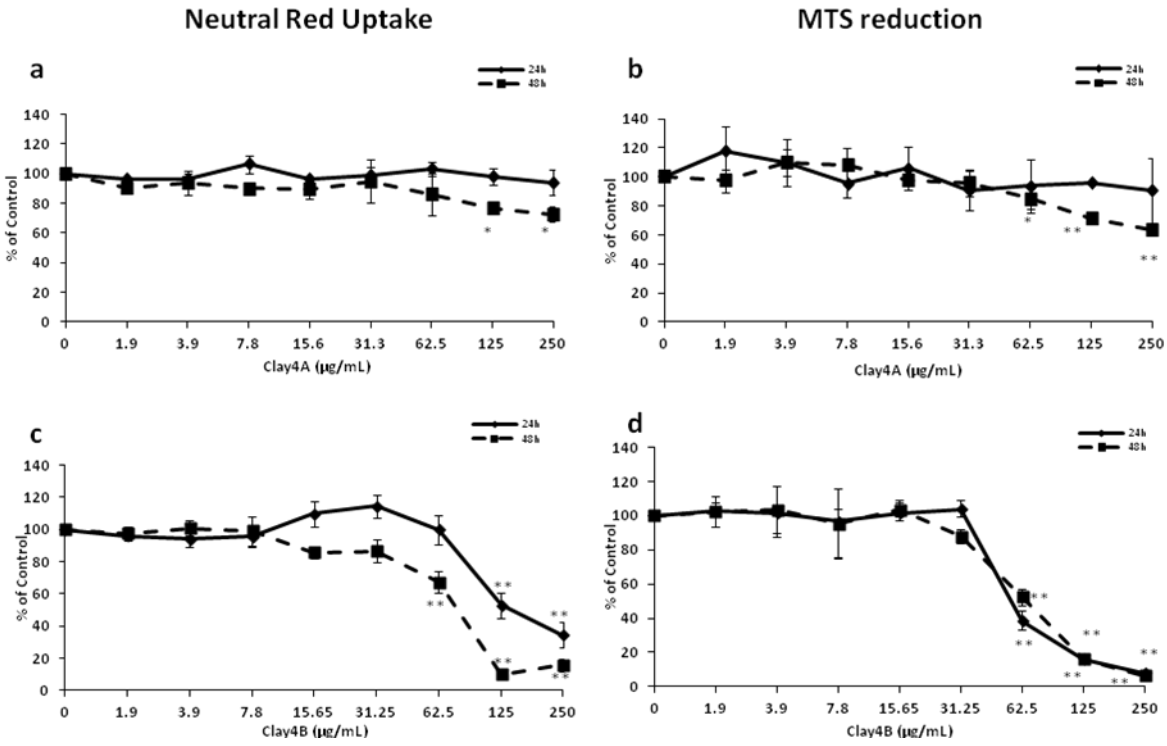

Figure 1. Neutral red uptake (a, c) and MTS reduction (b, d) of Caco-2 cells after 24 h and 48 h of exposure to 0–250 μg/mL Clay4A (a, b) and Clay4B (c, d). Results from 3 independent experiments with 6 replicates/experiment. All values are expressed as mean ± s.d. Differences were considered significant from $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Oxidative stress assays

When Caco-2 was exposed to Clay4A during 24 and 48 h, no significant alteration on GSH content was observed at any of the exposure concentrations in comparison to the control group (Fig. 2a). Caco-2 cells experienced a significant increase in ROS levels

when they were exposed from the concentration of 125 μg/mL Clay4A after 24 h, while this increase occurred with 62.5 μg/mL Clay4A after 48 h (Fig. 2b).

GSH content was not affected when Caco-2 was exposed to 10 or 20 μg/mL Clay4B (Fig. 2c) during 24 and 48h. In contrast, significant ($p<0.001$) reduced appeared at the highest concentration assayed in both times. The percentage of GSH for 40 μg/mL after 24 or 48h was 3.15 and 4.3 fold lower respectively, in comparison to that of the control group. (Fig.2c). Cells exposed to Clay4B did not show any change in GSH at any of the concentrations and exposure times assayed (Fig. 2d).

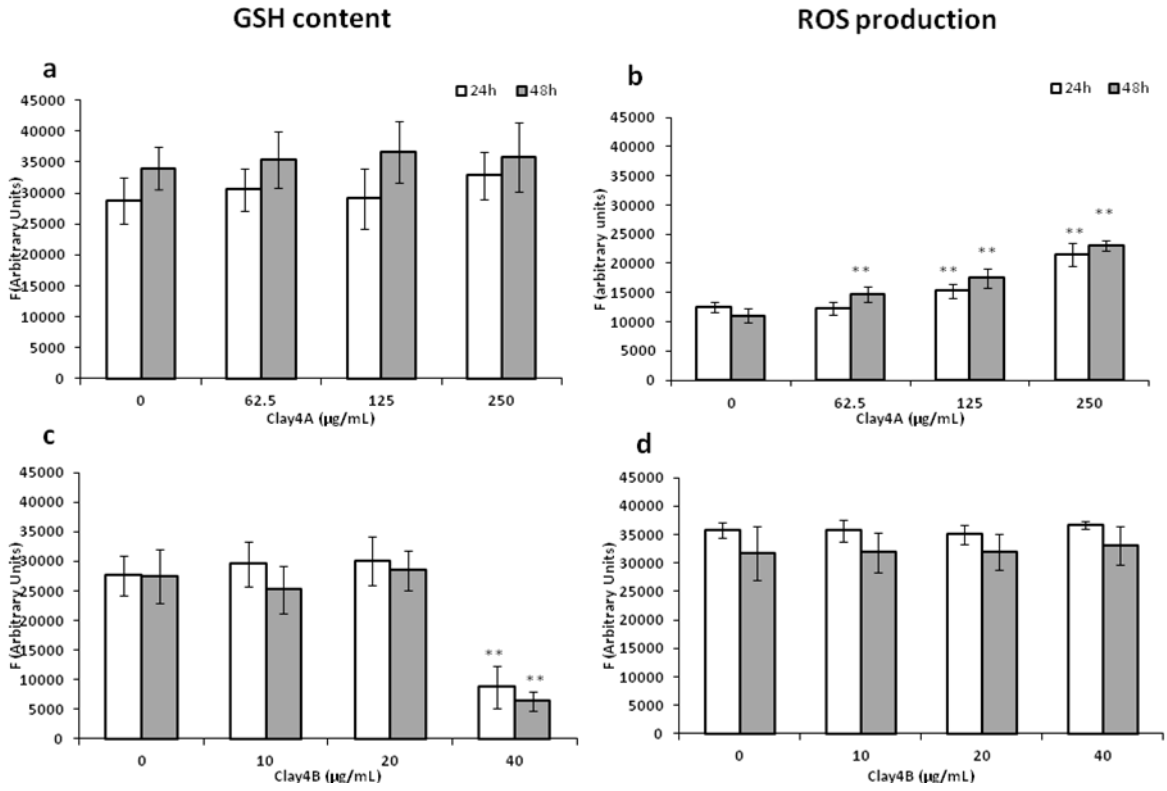

Figure 2. GSH content (a, c) and ROS production (b, d) in Caco-2 cells after 24 and 48 h of exposure to 62.5, 125 or 250 μg/mL Clay4A (a, b) or, 10, 20 or 40 μg/mL Clay4B (c, d). Results from 3 independent experiments with 3 replicates/experiment. All values are expressed as mean ± s.d. **significantly different from control ($p \leq 0.01$).

Comet Assay

The comet assay was performed in order to study the genotoxicity potential of the treatments in Caco-2. Clay4A (0–250 μg/mL) did not induce DNA strand breaks in Caco-2 cells at any concentrations tested after 24 or 48 h of exposure (Fig. 3a). Caco-2

cells exposed to 100 µM H_2O_2 (positive control), a significant ($p < 0.001$) increase of DNA damage was observed after 24 h and 48 h of exposure. However, when the Caco-2 cells were exposed to the highest concentration of Clay4B (0-40 µg/mL) for 24, a significan ($p < 0.001$) difference in DNA damage was observed. The per cent DNA in the tail at 40 µg/mL was approximately 7-fold higher compared to the other concentrations of Clay4B. In contrast, after 48h, a significant ($p < 0.001$) increase of DNA strand break was detected in cells exposed to 20 and 40 µg/mL (Fig 3b). Moreover, the damage was greater compared to 24 h ($p < 0.001$). The percentage of DNA in tail for 20 and 40 µg/mL was 15.5 and 30 fold higher respectively, in comparison to that the control group.

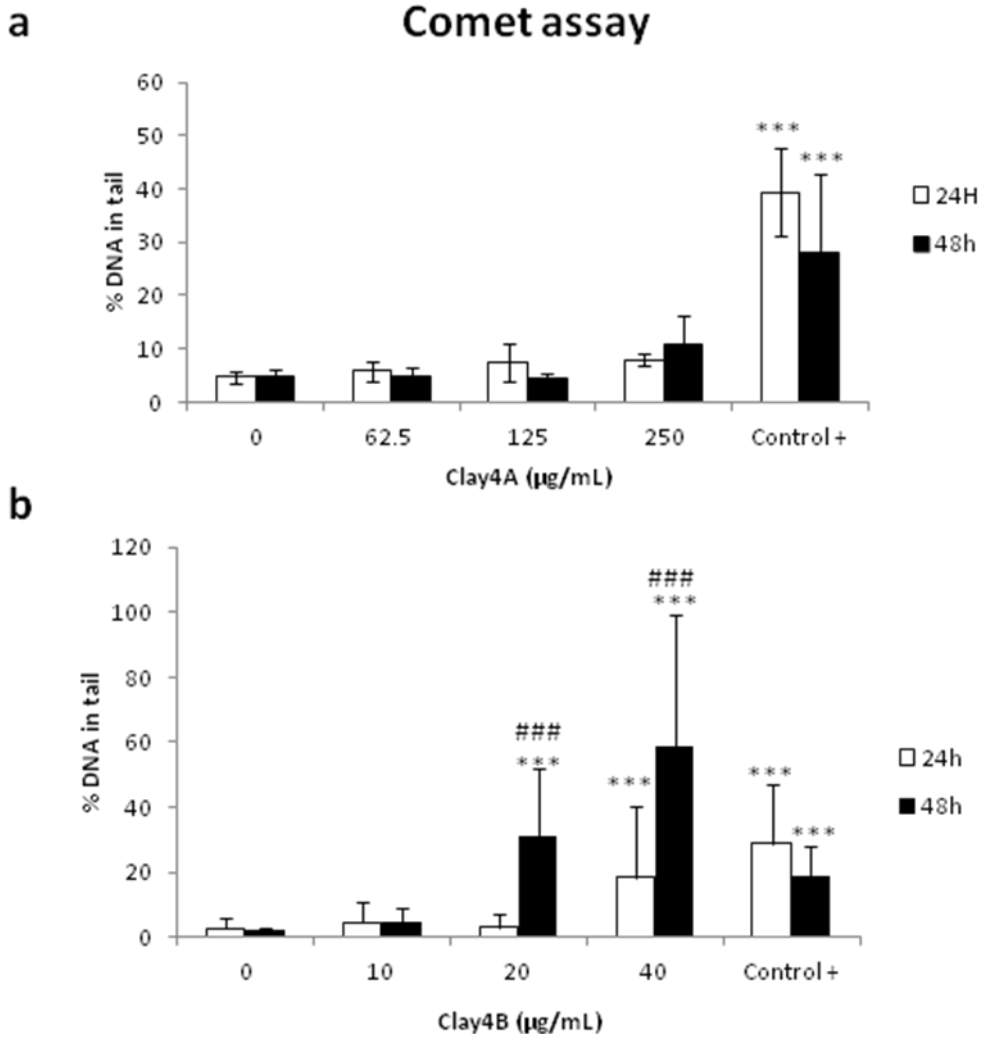

Figure 3. Comet assay results of Caco-2 cells after 24 and 48 h of exposure to 62.5, 125, or 250 µg/mL Clay4A (a) or, 10, 20 or 40 µg/mL Clay4B (b). Results from 3 independent experiments with 2

replicates/experiment. All values are expressed as mean ± s.d. *** very significantly different from control ($p \leq 0.001$) or ### groups exposed for 48h in comparison to 24h ($p \leq 0.001$).

Flow Cytometry

An annexin-V/PI double staining assay was performed to detect apoptotic and necrotic cells after exposure to different concentrations of Clay4A and Clay4B during 24 and 48 h. In the case of cells exposed to Clay4A (Fig. 4), a significant reduction of cell viability after 24 and 48 h was observed respect to the control group from the lowest concentration assayed (62.5 µg/mL). However, after 48 h, an increment of apoptotic cells was observed compared to cells exposed to Clay4A during 24 h. When cells were exposed to Clay4B (Fig. 5), a concentration-dependent increment of necrotic cells appeared after both exposure periods, although after 48 h this increment was more marked. Moreover, apoptosis was more intense in those cells exposed to 40 µg/mL after 24 h and in those cells exposed from 20 µg/mL after 48 h.

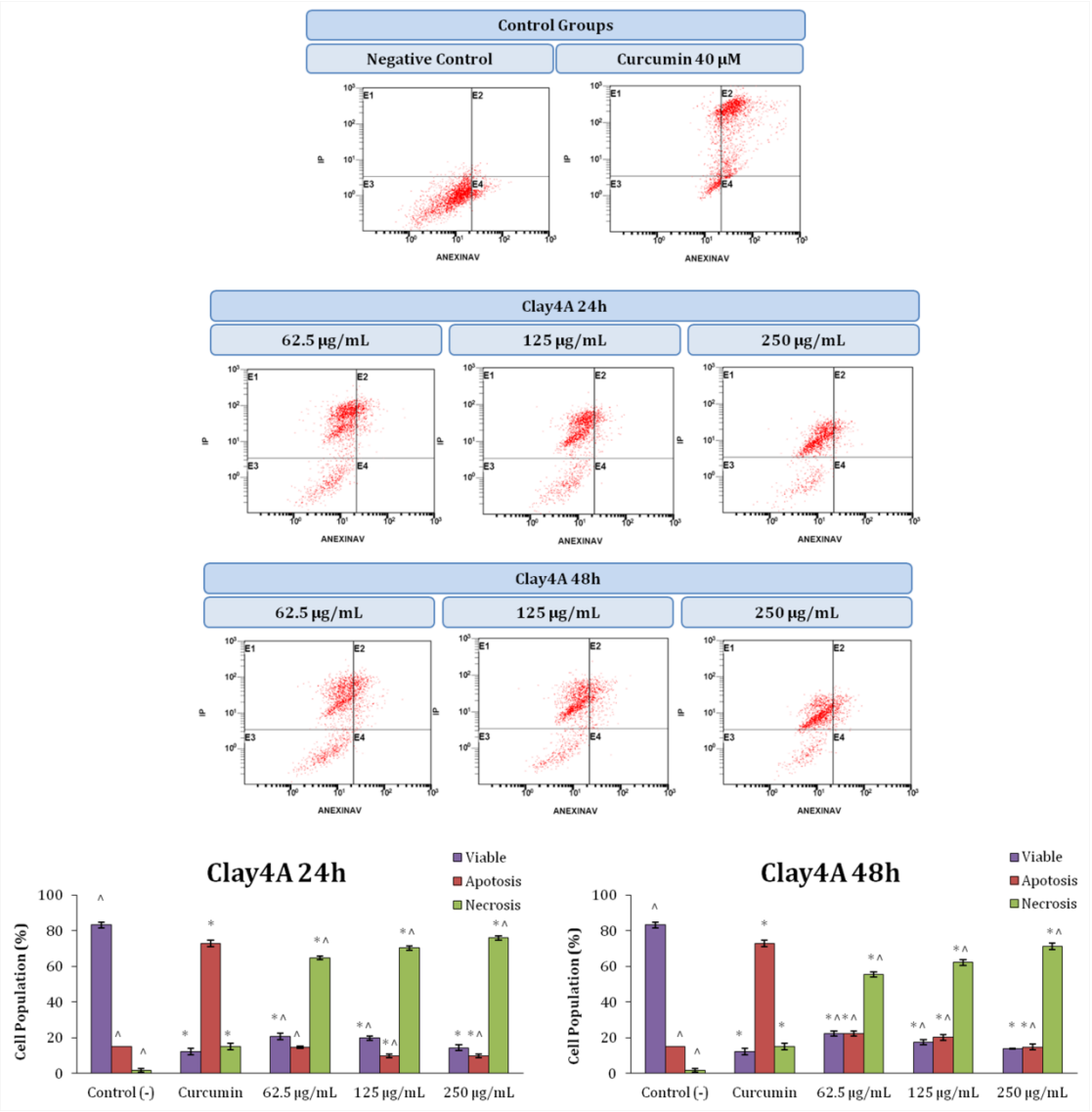

Figure 4. Viable (E3), early and late apoptotic cells (E4 and E2, respectively) and primary necrotic cells (E1) detected by flow cytometry after 24 and 48 h of exposure to 62.5, 125 and 250 µg/mL Clay4A. Moreover, a negative control and a positive control (Curcumin 40 µM) were used. * Significant respect to the negative control group ($p<0.001$). ^ Significant respect to the positive control group ($p<0.001$).

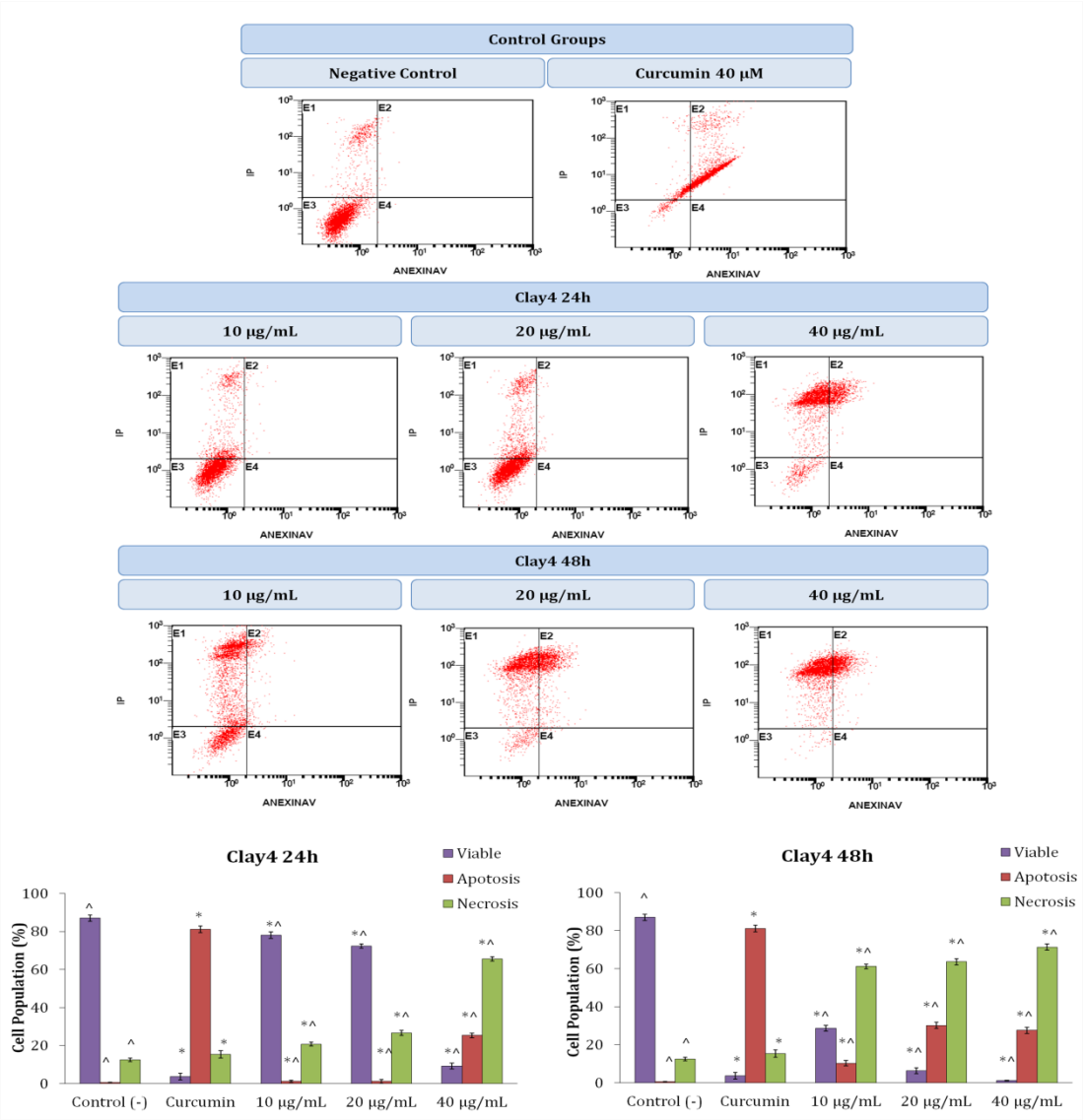

Figure 5. Viable (E3), early and late apoptotic cells (E4 and E2, respectively) and primary necrotic cells (E1) detected by flow cytometry after 24 and 48 h of exposure to 10, 20 and 40 µg/mL Clay4B. Moreover, a negative control and a positive control (Curcumin 40 µM) were used. * Significant respect to the negative control group ($p<0.001$). ^ Significant respect to the positive control group ($p<0.001$).

Metal content on the migration extracts

Clay4A migration extracts presented significant changes with respect the control in Al and Ca with ethanol 10% and, in Ca and Mg with isooctane.

	Al	Ca	Fe	Mg	Si
Ethanol 10% extract					
Control	25±7.8	110.5±19.1	0.4±0.3	7.3±2.5	12500.7±561.1
Clay4	45.7±8*	500±150*	0.7±0.3	18±7.1	14388±1572.9

Isooctane extract					
Control	60.3±5.5	275.7±65.6	42±7.1	20±4	4270.7±321.9
Clay4	74±15.6	445.5±2.1*	44±15.5	41.5±10.6*	4549.5±292

Table 1. Al, Ca, Fe, Mg and Si content in the migration extracts with etanol 10% or isooctane from PP and PP–Clay4 (all the concentrations are expressed in µg/L). All values are expressed as mean ± SD, n=3. *Significant ($p \leq 0.05$) and **very significant ($p \leq 0.01$) differences from control.

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CAPÍTULO 15/ CHAPTER 15

S. Maisanaba, M. Llana-Ruíz-Cabello, D. Gutiérrez-Praena, S. Pichardo, M. Puerto, A.I. Prieto, A. Jos, A.M. Cameán

NEW ADVANCES IN ACTIVE PACKAGING INCORPORATED WITH ESSENTIAL OILS OR THEIR MAIN COMPONENTS FOR FOOD PRESERVATION

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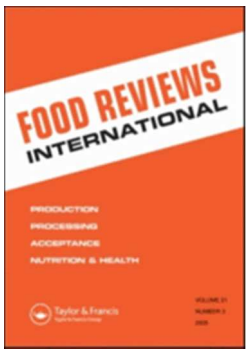

New advances in active packaging incorporated with essential oils or their main components for food preservation

Journal:	*Food Reviews International*
Manuscript ID:	Draft
Manuscript Type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Maisanaba, Sara; University of Sevilla, Llana-Ruiz-Cabello, María; University of Sevilla, Gutiérrez-Praena, Daniel; University of Sevilla, Pichardo, Silvia; University of Sevilla, Puerto, María; University of Sevilla, Prieto, Ana Isabel; University of Sevilla, Jos, Ángeles; University of Sevilla, Cameán, Ana María; University of Sevilla,
Keywords:	food processing/engineering < Food Science and technology, food microbiology/safety < Food Science and technology, shelf life < Food Science and technology

Active packaging for food preservation

New advances in active packaging incorporated with essential oils or their main components for food preservation

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D.*, Pichardo, S., Puerto, M., Prieto, A.I., Jos, A., Cameán, A.M.

Area of Toxicology, Faculty of Pharmacy, University of Sevilla. C/Profesor García González 2, 41012 Sevilla, Spain.

* Daniel Gutiérrez-Praena: Area of Toxicology, Faculty of Pharmacy, University of Sevilla. C/ Profesor García González 2, 41012 Sevilla (Spain).

Telephone: +34 954 556 762

Fax: +34 954 556 422

Email: dgpraena@us.es

Abstract

The food industry is developing new packaging systems (active packaging) through the incorporation of essential oils or their main compounds into the films. This could confer to them the antimicrobial and/or antioxidant properties already described for these substances themselves, improving the shelf-life of perishable food products. However, a safe range of concentrations for their use in active food packaging should be established to reach the market proposals but avoiding risks for population. The aim of the present work was to review the scientific literature concerning these two properties together with the effects induced by these substances on human cell lines.

Keywords: essential oil, active packaging, antimicrobial activity, antioxidant activity, cytotoxicity

1. Introduction

The food industry, in order to satisfy consumers, is developing new packaging systems, such as smart packaging, application of nanocomposites, use of biosensors, active packaging, etc, in agreement with "green consumerism" (1). In addition, there is also a trend directed to obtaining biodegradable packaging materials composed by cellulose, starch, poly-beta-hydroxyalkanoates, polylactide acid, etc, in order to reduce the using of non-biodegradable plastics (derived from petroleum) (2). Moreover, synthetic chemicals additives are being substituted by natural alternatives, since several findings have elucidated some problems concerning the safety of these chemicals, including allergenicity and carcinogenicity (3).

Food safety and quality are the two major concerns for food industry. Food-related microorganisms can lead to deterioration of foodstuff, but also can produce several diseases in humans (4). In this sense, the WHO has estimated that, each year, foodborne diseases affect 30% of people from industrialised countries, being diarrheal diseases the more severe (5-7). According to a recent report, the European Food Safety Authority (EFSA) in collaboration with the European Centre for Disease Prevention and Control (ECDC) estimated that 55453 European fell ill, 5118 got hospitalized, and 41 died in 2012 due to foodborne outbreaks (8). Among the microorganisms involved in foodborne diseases, the more common are *Staphylococcus aureus*, *Salmonella spp.*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Toxoplasma gondii*, *Bacillus cereus*, *Aspergillus niger*, and *Saccharomyces cerevisiae* (9-10). It is remarkably that *Salmonella spp.* with 31% food-related deaths, *L. monocytogenes* with 28%, *T. gondii* with 21%, *C. jejuni* with 5%, and *E. coli* O157:H7 with 3%, are the more harmful pathogens of the above mentioned (11). These microbes are usually found

in food of animal origin, particularly pork, beef, chicken, eggs, and milk, but also in fresh lettuce (12).

In addition to microbial growth, oxidation also plays a role in numerous food products. The oxidation may occur when reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals, singlet oxygen, and hydrogen peroxide, accumulate in tissues (13). Lipids (mainly), proteins, and carbohydrates are susceptible to oxidation, which lead to the alteration of different food organoleptic properties (colour, flavour, texture), or even become a nutritional risk (14).

Essential oils (EOs) are very interesting substances as they could substitute synthetic chemicals additives. This is justify by their antimicrobial and antioxidant properties, highlighting among some other properties as antitoxigenic, antiparasitic, insecticidal, antiphlogistic, spasmolytic, antinociceptive, immunomodulant, psychotropic, acaricide, expectorant, antidiabetic, cancer suppressive, etc (5; 15-16). These properties could be due to EOs *per se* or to the different compounds which are part of their composition. The EOs constituents are a wide group of molecules divided on the basis of their chemical structure (17-18). Depending on their structure, these compounds will have a related mechanism of action, which is interesting based on the type of activity required (18). In this sense, a previous review carried out by our group summarized the characteristics of the most common EOs used in food industry (19). Many EOs components are recognised by the Food and Drug Administration (FDA) of the United States of America as generally recognised as safe (GRAS) substances. In addition, not only these components appear in the list but also other EOs such as those obtained from clove, oregano, thyme, basil, and cinnamon (18; 20). Moreover, the European Commission authorised the use of some compounds from EOs (linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, limonene) as

flavourings in food products (21). On the contrary, no EOs or herbs extracts, with the exception of the rosemary EO, have been approved as additives for food by the European Commission (22).

Some of the most evaluated EOs components are cinnmaldehyde, eugenol, geraniol, thymol, menthol, and carvacrol (12; 23). Ortega-Ramírez et al. (10) stated that the antimicrobial and antioxidant properties of bioactive compounds are mainly due to their REDOX properties, ability to chelate metals, and quenching reactive species of singlet oxygen. For this reason, EOs or their compounds alone are a good option to be used in food industry for the improvement of shelf life of foodstuff. However, there are some limitations for the application of these substances since chemical variability could play an important role on their activity (13).

Solorzano-Santos and Miranda-Novales (12) pointed out that EOs or their compounds alone directly added to food, present desirable effects (antimicrobial and/or antioxidant) at certain concentrations, although these concentrations could produce undesirable changes in flavour, odour, etc. In order to avoid these effects, the use of packaging incorporated with EOs is an interesting alternative. These new systems receive the name of "active packaging", a novel concept where the packaging interact with food to increase the shelf-life of the later. Nowadays, the interest is trusted in biopolymers derived from recycling materials or renewable resources, including naturally occurring polysaccharides (where chitosan plays an important role), lipids, proteins from plants and animals, and those chemically-synthesized from monomers such as lactic acid (2; 24). However, the use of this kind of packaging in Europe and the ingredients used for this purpose are still not regulated by the EFSA, whereas in non-European countries as the USA, Japan and Australia this technology is approved (25).

The biological effects of many EOs intended for food preservation have been extensively reviewed. In this sense, Bakkali et al. (17) stated that EOs or their components induced cytotoxic effects in foodborne pathogens, mainly, through a prooxidant mechanism. Moreover, Llana-Ruiz-Cabello et al. (19) described in their review the more relevant mutagenic/genotoxic effects induced by EOs or their components in foodborne pathogens but also in human cell lines. However, to our knowledge, no other toxicological data such as cytotoxicity or morphological alterations in human cell lines have been recently brought together in a review. This would improve the understanding in this area as it could be another interesting tool to ensure the safety of EOs or their main components before being incorporated into food packaging. In addition, these mechanisms of action are related with the antimicrobial and/or the antioxidant properties of EOs or their main components, as we mentioned before, which could attribute active packaging interesting properties to become a real alternative in food preservation.

The present manuscript is divided into three sections. In the first one, an approach to the antimicrobial activity of EOs or their main compounds either alone or incorporated in active food packaging has been addressed. The second section deals with recent studies performed concerning the antioxidant activity (AA) of these mixtures or substances. Finally, the third section presents the state-of-the-art of the cytotoxic and morphological studies carried out in human cell lines with EOs or their main compounds. All the information presented below would make an interesting approach to an updated state-of-the-art of these new interesting active packaging systems as well as the risk associated to the possible commercialization of these new packages.

2. Antimicrobial activity

Below, is presented an approach to the antimicrobial characteristics of EOs and their main components *per se* and after their inclusion into film matrices intended to food packaging. Considering that previous works have already reviewed this activity, the present work, was focused on studies published between 2012 and 2015.

2.1. Antimicrobial activity of essential oils or their main constituents *per se*

The antimicrobial activity of EOs is of great interest, with more than 15000 reports available in the scientific literature including several reviews (5; 18; 26-28).

The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including bioautographic, diffusion, and dilution methods. Moreover, there are other less commonly used techniques such as turbidimetry or conductimetry, based on optical density evaluation or changes in the electrical conductivity, respectively (29). However, there are two methods which have been widely accepted and commonly used; agar disk diffusion and agar dilution. The agar diffusion method is a simple and commonly used method which basic principle is the diffusion of EOs through agar. An effective antimicrobial EO can inhibit the growth of microorganisms, producing an inhibition zone (15). The agar dilution method can be developed in two different ways: a) direct contact assay (agar or broth dilution method), where the microorganisms are first pre-cultured in a suitable growth broth and then, the bacterial suspensions are mixed with different dilutions of EOs and incubated under defined conditions depending on the microorganisms assayed, in order to know the Minimum Inhibitory Concentration (MIC) of the EO; b) vapor phase assay, which allows to evaluate the antimicrobial effectiveness of EOs in vapor phase through the addition of different concentrations of EOs to sterile filter disks placed on the lids of

Petri dishes, being an interesting method to evaluate antimicrobial active packaging (30). In this case, the MIC is determined as the lowest EO concentration which made a visible inhibition zone. These two methods together with turbidimetry, bioautography, or time killing assay have been discused by Seow et al. (27).

The results of these different antimicrobial tests often depend on several factors such as composition of active agents, plant differences, physical and chemical characteristics of the EO components, the presence of other substances like starch, proteins, or lipids, species and strain, the method used, culture conditions, etc (31). As reported by Seow et al. (27), other different factors as the type and volume of broth, temperature, time of incubation, and concentration and age of inoculums, also play a role in the EO antimicrobial potency.

Regarding the sensitivity of microorganisms to EOs, different authors suggested that Gram-positive (G+) bacteria are more susceptible to EOs or their components than Gram-negative (G-) bacteria (5; 27-28; 32). This could probably due to the outer membrane of G- bacteria, which contain hydrophilic lipopolysaccharides that create a barrier toward macromolecules and hydrophobic compounds, providing G- bacteria with a higher tolerance towards hydrophobic antimicrobial compounds from EOs (2; 33-39). However, recent studies reveal that, in particular cases, G+ bacteria exhibited more resistance than G- to EOs (2; 37; 40). In any case, the antibacterial activity of EOs is not referable to one specific mechanism, but a result from several damages in cells. These damages include membrane permeabilization or membrane disruption, leakage of cellular components, changes in the concentration of ATP, or enzyme inhibition. The study of bactericidal mechanisms of EOs has been reviewed by Hylgaard et al. (18) and Seow et al. (27). With respect to fungi, some authors reported than molds growth is

difficult to be inhibited, due to their complex structure (41). However, EOs appears to be more effective against yeasts and molds than for bacteria (5; 35; 42-43).

Despite the demonstrated antimicrobial potential of EOs and their constituents *in vitro*, their use as preservatives in food has been limited because high concentrations are needed to achieve sufficient antimicrobial activity (18). This may change the organoleptic properties of food resulting in palatability problems. For this reason, the development of active packaging containing EOs or their main constituents is an interesting strategy to solve this issue.

2.2. Antimicrobial activity of active packaging containing essential oils or their main constituents

The antimicrobial activity of films incorporated with EOs and their main components have been reviewed by several authors, proving the importance that these active packaging have acquired recently (26; 44-45). Thus, the oregano EO is the most frequently used for this purpose followed by cinnamon and clove EOs, taking into account the reviewed literature. Films containing the major compounds of EOs have been also studied, but advances in this field are scarce, and fewer reports are available in the scientific literature (46-48). The diversity of polymeric matrices studied is high but most of the studies are focused on the evaluation of the percentage of EO or the main compound required in the matrix to obtain the better antimicrobial properties, although there is no a comparison of different polymers with the same % EO or main compound. However, since the publication of these reviews, many other studies mainly concerning EOs incorporated to films have been carried out. This justifies the aim of the present review compiling studies on active packaging containing EOs, and it is summarized in table 1.

The antimicrobial activity of EOs has been attributed mainly to phenolic compounds, which have boiling points above 200 ºC. This fact makes these substances able to be incorporated into polymeric matrices for the development of antimicrobial active packaging, since they could be thermally processed without losing their properties (42). The advantage of applying EOs through the use of films, instead of applying them directly on foods, allows to attain the desired goal with lower oil concentrations, thus limiting unwanted flavors and odors to food (43; 49-50).

Once the EO is incorporated into the film, the antimicrobial activity needs to be assured. In this regard, the antimicrobial activity of these systems has been recently reviewed by Sadaka et al. (26), who reported that many factors need to be considered during the design, such as targeted microorganism, antimicrobial agent, chemical nature of food and antimicrobials, or packaging process conditions. The increasing interest about these natural sources has brought forward a new determinant factor, the film matrix, since it is necessary to reach a minimum concentration of the active compounds in the medium during the lag phase of the microorganisms to inhibit their growth, which occurs at a different liberation rate depending on the film matrix (51-52). Other authors have stated that these systems could also present synergistic effects. Thus, there are studies that observed differences in the antimicrobial activity of EOs included in film matrices against the same microorganism depending on the nature of this matrix (2; 35; 52-55). In addition, there are other reports that confirm these differences and also attributed these findings to different factors such as the resistance showed by collection strains and natural strains or the impaired penetration of EOs compounds in the products (43). For these reasons, it is very remarkable that Sung et al. (56-57) observed the same results for two different polymeric matrices containing the same amounts of garlic EO. In this regard, Avila-Sosa et al. (53) compared three different matrices (amaranth,

chitosan or starch edible films) with oregano and cinnamon EO and concluded that chitosan films exhibited the greatest inhibition against *Aspergillus niger* and *Penicillium digitatum*. Also, Otero et al. (52) observed that polyethylene terephthalate films incorporated with oregano EO presented a higher antimicrobial capacity than that presented by polypropylene films, also including oregano EO. Moreover, it has been demonstrated that the using of two or more EOs in active packaging improve their properties (58).

3. Antioxidant activity

The present section deals with the more recent advances concerning the AA of EOs and their main compounds *per se* and after the incorporation of them into different film matrices. Taking into account previous works in this field, this section have updated the information available in this concern from 2010 to 2015.

3.1. Antioxidant activity of essential oils or their main constituents *per se*

Antioxidant activity of EOs has been extensively described by many authors (59-63). This activity is due to their composition, rich in terpens, terpenoids, and aromatic compounds (17). The relatively recent interest of the consumers for natural products has lead to a renewal of scientific concern in these substances in detriment of synthetic antioxidants such as Butylated hydroxyanisol (BHA) and Butylhydroxytoluene (BHT) (5; 59).

There are many methods used for the antioxidant evaluation of the EOs or their main components. Alam et al. (64) described 29 methods for the *in vivo* and *in vitro* AA evaluation of chemical compounds in general; including those described by Amorati et al. (59) who reported 11 methods for specifically evaluate the AA of EOs. In addition,

Valgimigli and Pratt (65) stated that compounds reacting with radical species are not necessary antioxidants, unless they meet three requirements: a) the radical specie is a peroxyl radical, b) the reaction of the compounds with the radical is faster than the reaction of the radical with the substrate, and c) the products do not worsen the situation.

According to Sacchetti et al. (61) and Schlesier et al. (66), the AA of phytochemicals cannot be evaluated by a single method due to their complex reactive nature; therefore, at least two methods are recommended. Moreover, Amorati et al. (59) mentioned that it is a common practice in the literature to classify an EO as an antioxidant when it shows this activity in some methods, but not all of them, which can induce to confusion. Among the antioxidant methods, the most used in the literature are the DPPH (2,2-diphenyl-1-picrylhidrazyl) test, the TEAC (Trolox-Equivalent Antioxidant Capacity) test, and the TBARS (thiobarbituric acid reactive species). The DPPH and the TEAC tests are useful indirect methods for evaluating the AA of both hydrophilic and lipophilic substances, consisting in the measure of the color change of a solution by the action of antioxidant compounds, and ensuring a better comparison of the results (61; 67-68), whereas the TBARS method measure the formation of a colored adduct between thiobarbituric acid (TBA) and malondialdehyde (MDA), an end-product of the lipidic peroxidation (69). Amorati et al. (59) established that the best way to evaluate the AA of a compound is to measure the rate of oxidation of polyunsaturated lipids under certain conditions with or without the presence of the EO at issue. They also established that methods like DPPH or TEAC should be used for preliminary screening, and at any case, tests where the evaluation occurs in the absence of both radical species or oxidazable lipids (FRAP, Folin-Ciocalteu test, etc), should be avoided. In this sense, in table 2 there are 7 studies using the FRAP (Ferric Reducing

Antioxidant Power) method, and 6 studies employing the Folin-Ciocalteu test, 2 of which used the Folin-Ciocalteu test alone for the evaluation of the AA. Nevertheless, Sanches-Silva et al. (70) exposed that none of the methods mentioned could be recognized as the best one.

There are several studies concerning the AA of EOs which compare the potential antioxidant ability of different oils. Thus, Sacchetti et al. (61) compared the AA of thyme and rosemary EOs, together with other 10 EOs, through 3 different methods: DPPH test, β-carotene bleaching test, and luminol-photochemiluminiscence (PCL) test. Thyme resulted to be the most potent antioxidant, followed by rosemary. Teixeira et al. (63) found that EOs of citronella, clove, oregano, and thyme were capable to inhibit 50% of the radical-scavenging activity of DPPH, being the more potent the EOs from clove and oregano. By contrast, coriander and basil did not show AA. These results were also obtained after performing a FRAP test. Lee and Shibamoto (71) found that thyme, basil and rosemary presented high AA, whereas cinnamon presented the lowest AA when they performed two antioxidant lipophilic assays (aldehyde /carboxylic assay and conjugated diene assay). Bentayeb et al. (60), after performing an ORAC assay, found that oregano, clove, cinnamon, and thyme presented high AA, whereas basil and rosemary did not. In general, these differences could be due to the hydrophilic or lipophilic character of the components of the EO, the concentration used, or the nature and physicochemical properties of the EOs (72).

Concerning the main compounds of the EOs, Bentayeb et al. (60) established a correlation between the main components of the EOs and the AA of the oil, such as carvacrol in oregano, eugenol in clove and cinnamon, thymol in thyme, etc. In this concern, Ruberto and Baratta (69) analyzed about 100 pure components of EOs for their antioxidant effectiveness. They found that phenols, such as α-terpinene, γ-terpinene,

carvacrol, thymol, and eugenol, possessed the highest AA, whereas sesquiterpenes hydrocarbons and non isoprenoid compounds showed a low or absent AA. Although α-terpinene and γ-terpinene presente AA and are present in the EOs from oregano, coriander, rosemary, and thyme, the AA of these oils could not be due to them, since they are not their main components, although many studies propose that the AA of EOs also depends on secondary components, such as α-terpinene and γ-terpinene (59; 69).

3.2. Antioxidant activity of active packaging containing essential oils or their main constituents.

These EOs or their main compounds are being incorporated into food packaging in order to take advantage of their antioxidant properties, considering that oxidation is one of the major causes of food deterioration (62; 70). Sanches-Silva et al. (70) and Eça et al. (73) gathered together in their respective reviews some of the literature concerning the antioxidant role of many films containing EOs or their main components intended for food packaging. It is noteworthy that the extracts more cited in the literature for this purpose are green tea, oregano, and rosemary, as table 3 shows. This fact could be related to their rich composition in catechins, carvacrol/thymol, and phenolic compounds (camphor, 1,8-cineole, α-pinene, geraniol, etc.), respectively, which are known as potent antioxidants (74-76).

The aim of the present section is to provide a complete revision of the state-of-the-art related with the antioxidant capacity of active packaging incorporated with EOs and/or their main compounds for better foodstuff preservation. Tables 2 and 3 summarize the more recent advances in films incorporated with EOs or their main compounds, respectively, with special attention in the assays performed for the evaluation of their antioxidant efficiency as well as the type of film used.

The usual antioxidant assays described for EOs or their main components are not recommended when investigating the AA of films incorporated with these substances, since the manufacturing process could affect their composition. For this reason, only those assays based on the oxidation of polyunsaturated lipids provide meaningful results (59).

Green tea is being used in active packaging due to its antioxidant properties, which have been mainly attributed to the phenolic compounds present in its composition, especially catechins (77-79). Active packaging incorporated with green tea has been reported to delay lipidic oxidation in different food models, due to catechins are actively involved in the scavenging process of free radicals, as well as to the stimulation of transcription factors and mitogen-activated protein kinases-dependent cell cycle regulation (77; 80-81). Films incorporated with oregano and thyme EOs have shown different AA depending on the content in carvacrol and thymol, although it has been also demonstrated that there is a synergism between both components (59). As green tea extract, the AA of oregano and thyme is related with the retardation of lipid peroxidation through their potent radical-scavenging activity derived from their composition (82). Moreover, the AA of films added with basil, cinnamon, citronella, clove, lemongrass, and thyme have been also studied and summarized in the present work.

It is also remarkable that the majority of films included in the present section have shown an AA to a greater o lesser extent. In this sense, chitosan or gelatin films have showed AA, possibly due to the fact that free radicals can react with the residual free amino groups NH_2 of chitosan (83-84) or the peptide fraction from gelatin (85-86).

Concerning EOs-added films, their AA could vary depending on the EO used or on the composition of the films. Several authors have described a lower AA of films added with EOs compared to the oils alone (87-92). This fact could be due to interactions between the components of films and EOs, such as polyphenols and proteins, reducing the availability of the antioxidant compounds, and also could be due to the loss of volatile compounds during the films processing (91). Moreover, depending on the simulant used for the assays, the AA could also changes. Thus, López-de-Dicastillo (93) found that AA of films was simulant-dependent, that is, depending on the hydrophilic or lipophilic character of the EO, a simulant or another (water or ethanol) must be chosen for the evaluation of the AA of the incorporated-films. Finally, there are also studies where the AA of a film was assessed after a simulated digestion, resulting in a decreased of the activity compared with the activity of the original film (78). These authors attributed this finding to the transformation of the components from the extract during the digestion process or to an interaction between compounds and film.

Thus, the use of EOs or their main components in food packaging is a good alternative to the using of synthetic antioxidants such as BHA or BHT due to the refusal of consumers and to their possible toxic effects described at high concentrations (94). However, it is remarkable that any of the studies here mentioned involving a food matrix studied the organoleptic characteristics of these matrices, since EOs are known for their strong flavor and odor. For this reason, it should be also important to include this kind of parameters in new studies, in order to avoid controversial opinions from consumers. Moreover, studies concerning the safety of the EO added-films should be carried out in order to ensure their safe using in food packaging in the concentrations intended for this purpose.

4. Basal cytotoxicity assays, cell death and morphological studies induced by essential oils or their main constituents

Considering the properties presented in the above sections, it is important to highlight that the use of EOs and their main compounds as pure substances, as well as incorporated in food packaging applications should be limited. In order to reach the market proposals but not trigger risk to the population, the toxicological assessment is required. In this sense, *in vitro* cytotoxicity tests are useful to define basal cytotoxicity, directly related to cell death induction (95). These studies are needed in order to define the concentration range for further *in vitro* testing (genotoxicity, mutagenicity) and ensure the safety of these substances before being incorporated in food packaging. In this regard, an updated revision of the cytotoxic studies of EOs and its components used in food packaging has been carried out and described below (Tables 4 and 5).

Despite de traditional use of EOs, most of the studies concerning the toxicity are very recent. Almost the 65% of the studies selected for this chapter are dated from 2010, the rest are ranged from 2000 to 2010, with only 3 works referenced before 2000 (96-98). This finding may be due, in part, to the believed harmlessness that they have been conferred. However, the lately increase in the toxicity assessment of EOs is undoubtedly related to their new applications, hence, in the food industry, and particularly in food packaging.

It is interesting to point out that most of the experiments performed have been conducted in human cell lines, mainly HepG2 and Caco-2 cells, from hepatic and intestinal origin respectively, in order to assess the interest on the safety of these substances (98-110). In the reviewed literature, the majority of the cytotoxic studies are relative to EOs obtained from basil, garlic, oregano, rosemary, and thyme (Tables 4 and

5). The more extensively used assays are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT), the lactate dehydrogenase release assay (LDH), the trypan blue exclusion test, the neutral red uptake (NRU) and the total protein content assay (PC). The comparison between them is difficult since each one has been conducted with EOs containing different compositions. Moreover, the concentrations and times of exposure differed, as well as the cell line and the endpoints used.

The MTT assay has demonstrated to be, in general, more sensitive than other endpoints. In this sense, MTT cell viability of human lymphocytes exposed to palmarosa, citronella and lemongrass EOs as well as to geraniol and citral resulted more sensitive than that evaluated with the trypan blue dye exclusion method after 3 h of exposure (111). Also, Usta et al. (112) observed a higher sensitivity of the MTT assay (100% cell death) compared with the NRU and the trypan blue exclusion assays (20% cell death) in HepG2 cells exposed to linalool 2 µM. Similar findings were also observed in HT-29 and SH-SY5Y cells exposed to onion peel extracts (113) and to an aqueous extract of basil (114), respectively. Llana-Ruiz-Cabello et al. (110) also observed this fact (MTT assay vs. NRU and PC) in Caco-2 cells exposed to carvacrol, thymol, and their mixture. Thus, the higher sensitivity of the MTT assay may be related to the toxic mechanism of EOs which target mitochondria (17). In this sense, several studies have implicated mitochondria as one of the possible targets for EOs, initiating thus various biochemical events, such as enzyme inhibition or cell death through depolarisation of the mitochondrial membranes by decreasing the membrane potential (112). In this regard, Bakkali et al. (17) suggested that the permeabilization of outer and inner mitochondrial membranes leads to cell death by means of induction of apoptosis and/or necrosis. Although the mechanism of apoptosis induction has been largely

studied, these same authors reported that EOs alone or in combined treatments mainly induced necrosis rather than apoptosis.

It is important to point out that most of the reviewed studies are focused on assessing the toxicity of the main compounds from EOs. This may be related to the inconveniences in the use of EOs, since their lipophilic nature makes difficult to obtaining homogeneous exposure dilutions in cell culture media. In addition, their complex and variable composition complicate the interpretation of the results. In fact, the study of a single compound facilitates establishing a relationship between the damage observed and the toxic mechanism, including the mechanism of cell entering. In this regard, many authors have suggested that compounds from EOs, such as cinnamon and clove, can permeate the membranes due to their lipophilic nature and hence become accessible to various intracellular targets, including mitochondria (115). Linalool from coriander EO has been reported to be the responsible of the cytotoxicity of the oil (112; 116). Similarly, carvacrol and thymol induced higher damage than the oregano EO in HepG2 cells (104) although Savini et al. (103) reported that no single compound seemed to be responsible for the cytotoxic effects observed, but the whole extract of oregano. Sinha et al. (111) also evidenced the involvement in the toxicity induced by citral in lemongrass oil and geraniol in palmarosa oil in human lymphocytes. Similarly, the biological activity of green tea has been attributed to its polyphenols, in particular, to catechins (117). Moreover, despite the difficulty in comparing the results from different studies, a concentration and time-dependent decrease in cell viability have been frequently reported. In this regard, the cytotoxicity of basil extract analyzed by MTT assay was reported to be non toxic in HepG2 cells exposed up to 50 µg/mL for 24 h (101), and decreased the viability of SH-SY5Y cells exposed to 2000 µg/mL at the

same exposure time (114), and the viability of pancreatic cells exposed to 80 µg/mL up to 7 days (118).

In addition to cytotoxicity studies, the cell death evaluation and the most remarkable morphological features altered by EOs and their main compounds used or intended to be used in food packaging is of great concern, and studies related to this are summarised on Tables 6 and 7. The apoptosis induced by extracts of *Origanum majorana* in Jurkat cells has been related to an up-regulation of p53 protein levels and down-regulation of Bcl-2α (119). Similarly, Gu et al. (120) found that linalool-induced apoptosis might be associated with activation of p53 and cyclin-dependent kinase inhibitors. Moreover, water garlic extracts induced a p53/p21-dependent cell cycle arrest in G2/N phase and apoptosis in HepG2 cells (99). In this sense, Hong et al. (121) confirmed the p53-dependent pathway of apoptosis by reporting higher sensitivity in p53-wild type H460 cells than in the p53-null type H1299 non small cell lung cancer cells. In addition, diallyl sulfide (DAS)-induced apoptosis in ATC cells was associated with a decrease of Bcl-2 expression and with an increase of Bax expression, activation of caspase-9 and caspase-3, and mitochondrial release of cytochrome C (122). Similar findings were also observed in MDA-MB231 cells exposed to carvacrol (123) and in SH-SY5Y neuroblastoma cells exposed to limonene and linalyl acetate (124). In the case of thymol, the apoptosis induced in HL-60 cells involved both caspase dependent and independent pathways (125). This induction of apoptosis has been also related to the affectation in the homeostasis of Ca^{2+} in human glioblastoma cells (126). However, in the case of menthol, the induced cell death was not associated with Ca^{2+} influx pathways (113). In addition, rosemary extract also down-regulated 15 genes related to apoptosis in A2780 cells (127).

Apoptosis caused by EOs and their components, as we mentioned before, has been also observed by morphological visualization. Hence, electron microscopy revealed that morphological changes characteristic of apoptosis occurred in cells treated with DADS (96). Similarly, carvacrol induced morphological characteristic of apoptosis changes such as cell shrinkage, rounding of cells and membrane blebbing in MDA-MB231 cells (123) and intense vacuolization in the cytoplasm and release of the vacuoles in Caco-2 cells (110). Dusan et al. (100) performed a cell death assessment based on morphological changes on several EOs and its components. Thus, cinnamon and clove EOs and their major component eugenol had almost no cytotoxic effect at lower doses, oregano EO and its component carvacrol slightly increased the incidence of apoptotic cell death, and thyme EO presented relatively high cytotoxicity, which increased both apoptotic and necrotic cell death incidence, although its component thymol showed no cytotoxic effect at the used doses. Similarly, Stammati et al. (98) observed no signs of apoptosis in HepG2 cells induced by thymol at any of the concentrations tested, although a necrotic death pattern with cytoplasmic extrusion and no uptake of vital dye was observed. In contrast, the same morphological analysis suggested an involvement of carvone, carvacrol and cinnamaldehyde with apoptosis. Moreover, limonene and linalyl acetate produced cell shrinkage, cytoskeletal alterations, with necrotic and apoptotic cell death in SH-SY5Y cells (124).

Despite the great concern that all these effects on human cell lines arises, the cytotoxic effects as well as the induction of cell death and morphological death damage caused by EOs and their components on cancer cell lines have been considered a marker of their potential role as chemopreventive agents. Hence, the selective death of cancer cell lines have been studied with EOs from garlic (121; 128), basil (118) and green tea (129) among others. In this regard, the effect of *Origanum vulgare* extract on viability

of human normal cells (colonocytes and fibroblasts) and colon cancer Caco-2 cells, confirmed a selectivity, since normal colonocytes were unaffected by the presence of the extract while the cancer cell line under the same conditions of exposure was highly affected (103). Also, Singh and Katiyar (130) suggested that EGCG was cytotoxic on skin cancer cells, but not in normal skin cells. However, these compounds have not only exhibited toxic effects on cancer cells but also on cells with non-tumoral origin (111). Therefore, in order to use EOs in the food industry, especially in food packaging, their safety should be completely ensured.

Taking into account all the information given about the cytotoxic effects of EOs or their components, a safe range of concentrations for its use in active food packaging could be established and, subsequently, the development of antimicrobial and/or antioxidant films for food preservation.

5. Conclusion

In the recent years, an exponential increasing of studies concerning the antimicrobial or antioxidant role of food packaging films incorporated with EOs or their main components has been observed, mainly due to the controversy showed by synthetic preservatives. These studies demonstrate the usefulness of these systems both *in vitro* and *in vivo*, although results are not always conclusive. This could be due to different factors inherent to the EO, the film, the conditions, etc. In this sense, it is important to establish an appropriate concentration range for their uses, based on toxicity studies, to ensure the safety of the consumers. For this reason, studies of suitable materials for film manufacturing as well as the compatibility of the EOs or their main compounds and the films, are of great importance.

Acknowledgements

The authors wish to thank the Spanish Ministry of Science and Innovation (AGL2012-38357-C02-01) co-financed with FEDER funds and Junta de Andalucía (AGR-7252) for the financial support for this study.

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Table captions

Table 1. Antimicrobial activity of films incorporated with essential oils.

Table 2. Antioxidant activity of films incorporated with essential oils.

Table 3. Antioxidant activity of films incorporated with the main compounds from essential oils.

Table 4. Cytotoxic effects of the main essential oils intended to be used in food active packaging.

Table 5. Cytotoxic effects of the main components from essential oils intended to be used in food active packaging.

Table 6. Cell death evaluation and morphological changes of the main essential oils intended to be used in food active packaging.

Table 7. Cell death evaluation and morphological changes of the main components from essential oils intended to be used in food active packaging.

Table 1. Antimicrobial activity of films incorporated with essential oils.

EO	Species	Experimental model Microorganism	Food matrix	Film	Observations	References
Basil	*Ocimum basilicum*	*Aspergillus niger* *Penicillium expansum* *Rhizopus arrhizus* *Saccharomyces cerevisiae* *Saccharomyces fibuliger* *Bacillus subtilis* *Escherichia coli Salmonella enteritidis* *Staphylococcus aureus*	-	Pullulan films - 6, 12, 18, 24 and 30 mg/cm2	EO alone antimicrobial activity showed to be higher than films containing EO activity. 6 to 18 mg/cm^2 films inhibited the growth of G+ bacteria. *S. aureus* and *B. subtilis* were the most sensitive strains. Except for *P. expansum*, films did not inhibit moulds growth.	(131)
		Mesophilic bacteria counts *Rhizopus arrhizus*	Apples	Pullulan films - 12.0 g EO d.w./100 ml	Pullulan EO coating was found to offer low antibacterial protection. Regarding moulds, EO incorporation to pullulan coating caused total inhibition of the tested strain growth.	
	Ocimum basilicum	*Escherichia coli* *Staphylococcus aureus*	-	LDPE/EVA – 0.5, 2 and 4% wt	Film with EO did not reveal sufficient antimicrobial activity against the strains assayed. Pure EO (0.2 ml) was conducted to prove the antimicrobial activity and found to be highly efficient.	(132)
Cinnamon	*Cinnamomum verum*	*Aspergillus niger* *Penicillium digitatum*	-	Amaranth, chitosan or starch edible films – 0.25, 0.5, 0.75, 1, 2 or 4% EO	For amaranth films only high concentrations of EO (2%) exerted antifungal activity for both *A. niger* and *P. digitatum*. Starch films showed similar activity against both microorganisms (from 0.5%). Chitosan films exhibited the greatest inhibition against both microorganisms.	(53)
		Escherichia coli O157:H7 *Listeria monocytogenes* *Salmonella enterica* serovar *choleraesuis* *Staphylococcus aureus*	-	Pectin films– 7.3, 15.7, 36.1 g/L	Only films containing 36.1 g/L EO showed antimicrobial activity against: *E. coli* O157:H7 > *L. monocytogenes* > *S. aureus*. More inhibition was detected for *S. choleraesuis*.	(133)
			Fresh-cut peach	Pectin-cinnamon leaf oil coating – 7.3, 15.7, 36.1 g/L	At the end of storage (15 days at 5 ºC), the coated peaches with the highest EO concentration showed less microbial growth than controls.	

	Cinnamomun zeylanicum fortified with cinnamaldehyde (900mg/g)	*Aspergillus flavus* (0.1 mg/mL)	-	* - 0.025 mg/mL to 1.6 mg/mL	After 48 (72) h of incubation, non-growth was found at 0.1 mg/mL. This MIC was slightly dependent on the concentration of the fungal suspension.	(41)
			-	PET – 2, 4, 6 and 8% EO	4% and 8% EO films caused total inhibition for all suspensions (10^4, 10^5 and 10^6 CFU/mL). After extending the incubation period for two months, films maintained the antifungal activity. Material stored at 4 ºC for two months showed the same antimicrobial activity against *A. flavus* than fresh film.	
	EO (cinnamaldehyde 82.5g/100g)	*Eurotium amstelodami* (0.5g/ 100g) *Penicillium commune* (2g/ 100g)	-	* - 0.5 to 32g/100g EO	*E. amstelodami* showed to be more sensitive to cinnamon EO than *P. commune*. 0.8% EO film induced 91% inhibition.	(134)
			-	Cassava starch – 0.4, 0.6 and 0.8% EO	0.8% EO film produced 25% inhibition to *E. amstelodami*. However, even at minimum concentration applied into the film, cinnamon EO showed inhibition against both strains.	
	Cinnamomun zeylanicum	*Escherichia coli* *Listeria monocytogenes* *Staphylococcus aureus*	-	Alginate/Clay nanocomposite film – 0.5, 1 and 1.5 w/v	The highest level of EO led to a complete inhibition of *L. monocytogenes* growth for the first 3 days. Moreover, for *S. aureus* and *E. coli* this activity was observed for the first 6 days. Then, *E. coli* > *S. aureus*> *L. monocytogenes* increased its growth.	(4)
	Cinnamomun zeylanicum	*Escherichia coli* *Listeria monocytogenes* *Photobacterium phosphoreum* *Pseudomonas fluorescens* *Salmonella choleraesuis* *Shewanella putrefaciens* *Staphylococcus aureus* *Vibrio parahaemolyticus*	-	Agar film – 2g/100mL Sodium alginate film – 2g/100mL	Disc diffusion method revealed that *P. phosphoreum* was the most sensitive microorganism. Moreover, antimicrobial activity of agar films was 1.4-fold higher than sodium alginate films.	(2)
		Total bacteria counts Aerobic mesophiles Luminescent bacteria H_2S-producers	peeled shrimps	Agar film – 2g/100mL Sodium alginate film – 2g/100mL	For the *in vivo* assay, alginate films showed better activity than agar films. The antimicrobial activity of alginate films remained throughout the studied period and contributed to extend shelf life of peeled shrimps.	
	Cinnamomun	*Candida albicans*	-	* – 0.8 and 1.5% EO	Film forming solution exhibited considerable	(37)

	zeylanicum	*Bacillus subtilis* *Escherichia coli* *Lactobacillus lactis* *Listeria monocytogenes* *Pseudomonas putida* *Streptococcus agalactiae*			inhibition against most of the strains. The lower activity of this solution was observed for *S. agalactiae* while *B. subtilis* was the most sensitive bacteria.	
			-	Whey protein edible film – 0.8 and 1.5% EO	Results for films were similar to that for film forming solution; however, inhibition zones were smaller than those on the film forming solution.	
	Cinnamon EO and rhubarb ethanol extract	Total viable counts	beef	PP/PVA containing both REE (0-4 v/v) and Cinnamon EO (0-0.32 v/v)	All treatments with antimicrobial films maintained better quality in comparison to the control. Higher concentrations of natural extracts inhibit bacterial growth more efficiently.	(135)
	Cinnamon zeylanicum fortified with cinnamaldehyde (900mg/g)	*Aspergillus flavus*	-	*	Cinnamon EO in vapour phase produced a significant reduction in Aflatoxin B1 production.	(136)
			-	PP – 2, 4 and 6% EO	PP containing 2% EO showed a significant reduction in Aflatoxin B1 production while PP containing 4 and 6% EO produced a total inhibition.	
	Cinnamomun zeylanicum	*Escherichia coli* *Staphylococcus aureus*	-	LDPE/EVA – 0.5, 2 and 4% wt	Films with 2 and 4 % EO almost completely inhibit both strains.	(132)
	Cinnamon EO	Lactic acid bacteria *Enterobacteriaceae* *Pseoudomonas* spp.	Chilled pork	Low densisty polyethylene films coated with 112 to 527 nm cinnamon EO nanoparticles (C-NPs)	The bacterial counts resulted in a significant increment with the storage time increasing (15 days). However, all counts were lower with the concentration increasing of C-NPs, being the 527 nm C-NPs the best ones preserving the samples.	(137)
	Cinnamon EO and cinnamon EO-nanoliposomes	*Aspergillus niger* *Escherichia coli* *Staphylococcus aureus*	-	Fish gelatin film – 5% v/v	Although at short time storage, gelatin-EO films showed better antimicrobial effects, the nano-active film indicated better control of pathogen after storage for one month. EO-nanoliposomes improved antimicrobial stability of films and prolonged the antimicrobial time.	(138)
	Cinnamon bark oil	*Salmonella thyphimurium*	Cherry tomatoes	Zein-based coating – 5 to 20% EO	Zein with 20% cinnamon EO achieved a more than 5 log CFU/g reduction of *S. Typhimurium* compared with initial control tomato samples.	(139)
	Cinnamon bark oil (CBO) (*Cinnamomun cassia*)	*Escherichia coli* *Listeria monocytogenes* *Salmonella enterica*	-	Sodium alginate film – 1 and 2% CBO	After 24 hours, films with CBO presented larger inhibition zones for *L. monocytogenes*. After 48 hours all inhibition zones drecreased, being more marked for *L. monocytogenes*. Overall, *E. coli* and *S.*	(140)

					enterica resulted more sensitive to this film. No differences were observed between different concentrations of CBO in films.	
Clove		*Aspergillus niger* *Debaryomyces hansenii* *Penicillium expansum* *Aeromonas hydrophila* *Bacillus cereus* *Bacillus coagulans* *Bifidobacterium animalis subespecies lactis* *Bifidobacterium bifidum* *Brochothrix thermosphacta* *Citrobacte freundii* *Clostridium perfringens* *Enterococcus faecium* *Escherichia coli* *Lactobacillus acidophilus* *Lactobacillus helveticus* *Listeria innocua* *Listeria monocytogenes* *Photobacterium phosphoreum* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Salmonella cholerasuis* *Shewanella putrefaciens* *Shigella sonnei* *Staphylococcus aureus* *Vibrio parahaemolyticus* *Yersinia enterocolitica*	-	Sunflower protein (SFP) film – 0.75 mL/ g protein	Antimicrobial activity against all the strains assayed was observed in different degrees. The yeast *D. hansenii* presented the highest inhibition while *C. perfringens* was the lowest inhibited microorganism.	(43)
	Syzygium aromaticum	*Escherichia coli* *Listeria monocytogenes* *Staphylococcus aureus*	-	Alginate/Clay nanocomposite film – 0.5, 1 and 1.5 w/v	The highest level of EO led to a complete inhibition of *L. monocytogenes* growth for the first 7 days. Moreover, for *S. aureus* and *E. coli* this activity was observed for the first 6 days although the	(4)

					antimicrobial effectiveness decreased afterwards in the case of *E. coli*.	
	Eugenia caryophyllata	*Aspergillus niger* *Candida albicans* *Penicillium citrinum* *Bacillus cereus* *Enterococcus faecalis* *Escherichia coli* *Salmonella enterica* *Salmonella typhimurium* *Staphylococcus aureus* *Streptococcus mutans*	-	Cassava bagasse-PVA biodegradable trays - 6.5, 8.5 and 10% w/w for direct incorporation (DI) and 2.5, 5 and 7.5% w/w for surface coating (SC)	The trays were more effective against moulds and yeasts than to bacteria. For DI assay, antimicrobial activity of films was observed only for *C. albicans*, while SC assay showed that films were effective against all microorganisms from 5% EO in films. Any concentration of EO in films showed total inhibition of strains.	(42)
	Eugenia caryophyllata (71.80 % eugenol)	*Enterobacteriaceae* Lactic acid bacteria *Pseudomonas spp.* Total aerobic mesophilic bacteria Total aerobic Psychrotrophic bacteria	Chicken breast fillets	WPI – 10 or 20 g /Kg	The higher EO concentration in the formulations, the higher the effectiveness of coatings. Clove coating exhibited lower activity than oregano coating. Antimicrobial edible coatings proved to be much more effective on chicken breast than the use of the direct addition of EO.	(141)
				EO – 20 g/Kg	A detrimental effect was observed with clove treatment. Significantly higher microbial counts were detected in treated samples compared with the control ones.	
		Bacillus cereus *Escherichia coli* *Pseudomonas fluorescens* *Staphylococcus aureus*	-	CMC/PVOH – 1, 2 and 3% EO	Films did not show any activity against G- bacteria (*E. coli* and *P. fluorescens*). Films with 3% EO showed the best antimicrobial activity.	(54)
			Ground chicken meat	CMC/PVOH – 3% EO	Shelf life extension was observed during refrigerate storage due to the antimicrobial effect of films containing clove EO. Chicken samples inoculated with *S. aureus* experienced a total elimination of bacteria from 7 days after packaging in 3% clove EO films.	
		Escherichia coli O157:H7 *Listeria monocytogenes*	-	Chicken feather protein film – 0.5, 1 and 1.5% EO	Results of disc diffusion method indicated strong antimicrobial properties of clove EO films against both bacteria, being more significantly for films with 1.5% of EO to *L. monocytogenes*.	(142)

			Smoked salmon	Chicken feather protein film – 0.5, 1 and 1.5% EO	Films with 1.5% EO showed a strong inhibitory effect against both strains.	
		Listeria monocytogenes *Staphilococcus aureus* *Escherichia coli* *Salmonella enteritidis*	-	Chicken feet proteins (5%) – 1% EO	Films presented a better antimicrobial activity against Gram-positive bacteria.	(143)
			Sliced cheddar cheese	Chicken feet proteins (5%) – 1% EO	Samples wrapped with the control films presented an exponential growing of microorganisms, while samples wrapped with the film containing clove EO presented a lower number of microorganisms.	
	Eugenia spp.	*Bochothrix thermosphacta* *Escherichia coli* *Listeria innocua* *Listeria monocytogenes* *Pseudomonas putida* *Salmonella typhimurium* *Shewanella putrefaciens*	-	Fish protein film-0.1 µL EO/cm^2 film	No antimicrobial activity was found for clove EO films with the agar diffusion method. However, these films showed antimicrobial activity in macrodilution assay only for both strains of *Listeria*. Moreover, clove EO films exhibited the highest antibacterial activity against *S. putrefaciens*.	(91)
	Syzygium aromaticum	*Escherichia coli* *Staphylococcus aureus*	-	LDPE/EVA – 0.5, 2 and 4% wt	Films with higher amount of EO showed better antimicrobial activity. Moreover, *E. coli* appeared to be more sensitive.	(132)
Coriander	*Coriandrum sativum*	*Brochothrix thermosphacta* *Escherichia coli* *Listeria innocua* *Listeria monocytogenes* *Pseudomonas putida* *Salmonella typhimurium* *Shewanella putrefaciens*	-	Hake protein edible film – 0.25 mL/g protein	Films containing EO did not showed inhibition in the agar diffusion method. However, in macrodilution method results showed inhibition only for *L. innocua* and *S. putrefaciens*.	(90)
		Escherichia coli *Listeria monocytogenes*	-	Porcine protein film – 0.8, 1, and 1.5% EO	The antimicrobial activity increased with the increment of EO incorporated into the films. Moreover, the inhibition was more significant for L. *monocytogenes* than for *E. coli*.	(144)
Garlic	*Allium sativum*	Total aerobic counts	Shrimps	Chitosan coating incorporated with garlic EO – 0.5, 1 and 1.5% (w/v)	Coating samples, even with chitosan only, improved the shelf life of shrimps, being 7 days for uncoated samples and 9 days for chitosan coated samples. The combined effect of garlic EO and chitosan coatings resulted in a longer storage time (11 days).	(145)
	Allium sativum	*Brochothrix thermosphacta*	Ready-	LDPE – 2, 4, 6 and 8%	For *L. monocytogenes*, all active films showed	(56)

		Escherichia coli *Listeria monocytogenes*	to-eat beef loaves	EO w/w	growth suppression, thus 2% EO was sufficient to suppress the growth. For *B. thermosphacta* and *E. coli* the antimicrobial effect was consider not effective since differences in counts between controls and films were low at <0.3 log cfu/g and <0.2 log cfu/g, respectively.	
	Allium sativum	*Brochothrix thermosphacta* *Escherichia coli* *Listeria monocytogenes*	-	LDPE/EVA – 2, 4, 6 and 8% EO w/w	2-4% EO films produced not obvious inhibition zones. However, a large retraction zone was observed for 6% EO films to *L. monocytogenes* and for 8% EO films to *B. thermosphacta* and *E. coli*.	(57)
			Cooked beef	LDPE/EVA – 2, 4, 6 and 8% EO w/w	For *L. monocytogenes*, all active films showed growth suppression, thus 2% EO was sufficient to suppress the growth. For *B. thermosphacta* and *E. coli* the antimicrobial effect was consider not effective since differences in counts between controls and active films were low at <0.3 log cfu/g and <0.2 log cfu/g, respectively.	
		Brochothrix thermosphacta *Escherichia coli* *Listeria innocua* *Listeria monocytogenes* *Pseudomonas putida* *Salmonella typhimurium* *Shewanella putrefaciens*	-	Fish protein film-0.1 µL EO/cm^2 film	The agar diffusion method revealed only a slightly inhibition for *Listeria monocytogenes*; however, macrodilution method revealed inhibition for *B. thermosphacta*, both strains of *Listeria* and *S. putrefaciens*.	(91)
Oregano	*Lithobates berlandieri*	*Aspergillus niger* *Penicillium digitatum*	-	Amaranth, chitosan or starch edible films – 0.25, 0.5, 0.75, 1, 2 or 4% EO	For amaranth films only 4% EO exerted antifungal activity and only against *A. niger*. Starch films showed better activity against *P. digitatum* (from 0.5% EO) than that for *A. niger* (from 2%). Chitosan films exhibited the greatest inhibition against both microorganisms.	(53)
	Origanum vulgare	*Escherichia coli* *Listeria monocytogenes* *Salmonella enteritidis* *Staphylococcus aureus*	-	Alginate films – 0.5, 1 and 1.5% w/v	Alginate films showed antibacterial effect after the incorporation of 1% EO. As the concentration of EO increased in the alginate films, the zone of inhibition also increased significantly. Alginate films containing EO were more effective against G+ bacteria. The inhibitory effects of alginate films	(146)

					containing EO was lower than that found for pure EO (data no shown)	
		Escherichia coli *Pseudomonas aeuginosa* *Staphylococcus aureus*		Triticale protein film – 1 and 2% w/v	Higher concentration of EO showed larger inhibition zones. G+ was more sensitive for these films. *P. aeruginosa* was the most resistant strain assayed.	(147)
		Listeria monocytogenes (30 µL/mL)	-	*	30 µL/mL of EO produced a clear inhibition zone against *L. monocytogenes.*	(38)
			Cold-smoked salmon	Potato peel waste-based edible film- 0.97 and 1.92% EO (185 and 286 mg oil/g film)	Films containing 289 mg oil/g film showed the highest antilisterial activity. Growth depression of *L. monocytogenes* on cold-smoked salmon during storage (4 ºC for 28 days).	
	Zataria multiflora	*Escherichia coli* *Listeria innocua* *Pseudomonas spp.* *Salmonella enterica* *Staphylococcus aureus*	-	Paper - 2, 4 and 6% w/w)	All bacteria were inhibited with 4% to 6% films. 2% films only inhibited *Staphylococcus aureus* and *Salmonella entérica*.	(148)
	Origanum vulgare	*Aspergillus niger* *Candida albicans* *Penicillium citrinum* *Bacillus cereus* *Enterococcus faecalis* *Escherichia coli* *Salmonella enterica* *Salmonella typhimurium* *Staphylococcus aureus* *Streptococcus mutans*	-	Cassava bagasse-PVA biodegradable trays - 6.5, 8.5 and 10% w/w for direct incorporation (DI) and 2.5, 5 and 7.5% w/w for surface coating (SC)	The trays were more effective against moulds and yeasts. G+ bacteria were more sensitive than G-. The lowest activity was observed for *E. faecalis* and *S. mutans*. These bacteria are able to form a biofilm that can reduce the antimicrobial effect of the EO. Antimicrobial activity stability of films showed that inhibition zones against *S. aureus* and *E. coli* were not altered during 15 days of storage. The largest inhibition zones were observed for the trays produced by SC method	(42)
	Coridothymus capitatus (48.56 % carvacrol)	*Enterobacteriaceae* Lactic acid bacteria *Pseudomonas spp.* Total aerobic mesophilic bacteria Total aerobic psychrotrophic bacteria	Chicken breast fillets	WPI – 10 or 20 g /Kg	The higher EO concentration in the formulations, the higher the effectiveness of coatings.Antimicrobial edible coatings proved to be much more effective on chicken breast than the use of the direct addition of EO.	(141)
				EO – 20 g/Kg	Direct addition of EO did not show inhibitory effects against microbial development. Lack of effectiveness was detected in most cases.	
		Enterobacteriaceae	Rainbow	Quince seeds mucilage	Films with 1 and 1.5% EO did not show any	(149)

		H_2S-producer bacteria Lactic acid bacteria *Pseudomonas spp.* Aerobic and psychrotrophic count Total viable counts	trout fillets	films – 1, 1.5 and 2% EO (v/v)	remarkable activity. Moreover, 2% EO films showed lower counts significantly different only for psychrotrophic count.	
	Zataria multiflora	*Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	-	Gelatin films – 2, 4, 6 and 8% w/w	All films containing EO showed antibacterial activity. G+ bacteria were more sensitive than G-. Higher amounts of EO in films showed larger inhibition zones.	(150)
		Moulds and yeasts *Enterobacteriaceae* Lactic acid bacteria *Pseudomonas spp.* Total viable counts	Foal meat	PET/PE/EVOH/PE-1.5-2 g/m^2	Although all microorganisms counts studied gradually increased along storage time (0, 4, 7, 10 and 14 days) at 2ºC, *Enterobacteriaceae* and moulds and yeasts showed more sustained growth. Significant differences between samples and control were found from day 10, but *Pseudomonas spp.* showed statistical differences from day 7.	(151)
	Origanum vulgare	*Escherichia coli* O157:H7 M364VO (400 mg/L) *Escherichia coli* O157:H7 CECT 5947 (200 mg/L)	-	PP and PET – 0, 4, 6 and 8% EO	In the vapour phase assay, oregano EO only inhibited partially or totally the growth of *E. coli* from 6 or 8%. Moreover, for films, higher concentration of EO in the active coatings resulted in higher antimicrobial activity, being 6% the minimum concentration showing inhibition against both strains. In direct contact assay PET films showed stronger *in vitro* antibacterial activity than films of PP.	(52)
			Ripened sheep cheese	PP and PET – 0, 4, 6 and 8% EO	4% EO PP films decreased counts of both strains, however films with 6 and 8% EO did not significantly reduce the growth. PET films antimicrobial capacity was greater than that of PP films.	
	Zataria multiflora	*Bacillus cereus* *Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhimurium* *Staphylococcus aureus*	-	Soluble soybean polysaccharide – 1, 2 and 3% oil v/v	No inhibition area was observed in the vapour-phase assay for films with 1% EO. All films inhibited the growth of the five tested bacteria in the overlay assay except *P. aeruginosa*, which was not inhibited at the lowest concentration.Vapour-phase test indicated that the effective levels of EO added to film were higher	(152)

					than those for the overlay assay.	
	Thymus capitatus	*Bochothrix thermosphacta* *Escherichia coli* *Listeria innocua* *Listeria monocytogenes* *Pseudomonas putida* *Salmonella typhimurium* *Shewanella putrefaciens*	-	Fish protein film-0.1 µL EO/cm^2 film	The agar diffusion method revealed only inhibition for both *Listeria* strains; however, macrodillution method revealed inhibition for *Bochothrix thermosphacta* and *L. innocua*. The addition of EO to films did not reduce the growth of G- bacteria.	(91)
		Bacillus subtilis *Escherichia coli* *Salmonella enteritidis* *Staphylococcus aureus* *Shigabacillus*	-	*	Oregano EO showed to be effective against all the strains assayed. *S. aureus* was the most sensitive bacteria, while *S. enteritidis* presented the smaller inhibition zone. However, results for all strains were slightly different.	(39)
			-	Gelatin (from silver carp skin)/chitosan – 1, 2, 3 and 4% EO v/v	Films presented antimicrobial activity when concentration of EO in film was higher that 1%. Greater inhibition zones were observed with higher concentrations of EO.	
	Zataria multiflora	*Bacillus cereus* *Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhimurium* *Staphylococcus aureus*	-	CMC – 1, 2 and 3 % v/v	Significant inhibitory effects on both G+ and G- bacteria. Effects increased with increasing EO concentration.	(153)
		Staphylococcus aureus *Listeria monocytogenes* *Salmonella enteritidis* *Escherichia coli*	-	Fish gelatin-Chitosan (2%-1.5%) – 0.4, 0.8, and 1.2% EO w/v	Films incorporated with 1.2% oregano EO presented the highest antimicrobial activity against all 4 pathogens studied. Among them, S. aureus appeared to be the most sensitive and E. coli the most resistant.	(154)
		Penicillium expansum *Escherichia coli* *Listeria monocytogenes*	-	EVOH – 5% w/w	Strong effect against fungal growth was observed. Regarding bacteria, agar diffusion method in vapour phase revealed higher inhibition for *E. coli*. For liquid media assay total inhibition of both strains was observed.	(79)
		Escherichia coli *Staphylococcus aureus*	-	Bovine hide gelatine (5%) EO 0-6000 ppm	Both microorganisms where sensitive to the film incorporated with the EO, being the inhibition higher with the EO content increasing in the film. *E. coli* appeared to be more sensitive than *S. aureus*.	(92)
Pennyroya		*Bacillus cereus*	-	Soluble soybean	No inhibition area was observed in the vapour-phase	(152)

l		*Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhimurium* *Staphylococcus aureus*		polysaccharide – 1, 2 and 3% oil v/v	assay for films with 1% EO and 2% EO only formed an inhibition zone for G+ bacteria. Films containing 3% EO did not show antimicrobial activity against G- bacteria in vapour phase assay. However, vapour-phase test indicated that the effective levels of EO added to films were higher than for the overlay assay.	
Rosemary		*Aspergillus niger* *Escherichia coli*	-	Starch-sodium alginate - 0.3, 0.6, 0.9 and 1.2% (w/w)	Results showed antimicrobial activity of all films against *E. coli* in a concentration dependent way. However, no activity was observed against *A. niger* in any film.	(155)
		Escherichia coli O157:H7 *Listeria monocytogenes* *Salmonella typhimurium*	Fresh broccoli	MC films PCL/alginate films	The antimicrobial effects on the growth of different microorganisms in broccoli during 12 days of storage at 4 ºC of two formulations (Acetic acid+rosemary extract+Asian EO (a) and Acetic acid+rosemary extract+italian EO (b)) were studied. Results showed that PCL/ALG- had strong, stable antimicrobial properties against *L. monocytogenes* compared to the others films. However, MC films were more effective against *E. coli* and *S. typhimurium* in mid- and long-term storage, being *S. typhimurium* the most sensitive microorganisms to these films.	(55)
		Listeria monocytogenes	-	* - 0.1, 0.2, 0.4 and 0.5% EO (v/v)	Rosemary EO with 0.5% (v/v) proved to be bactericidal after 48 h storage at 10 ºC	(135)
		Lactic acid bacteria *Listeria monocytogenes* Total aerobic counts	Mozzarella cheese	Nylon/EVOH/PE bag with paper sachets containing thyme EO – 1% EO	Results showed that sachets containing 1% EO did not exert strong antilisterial activity, but slower growth and lower counts of *L. monocytogenes* were observed. However, the growth of LAB and TAB decreased with this treatment.	
Thyme	*Thymus vulgaris*	*Brochothrix thermosphacta* *Enterobacteriaceae* Lactic acid bacteria *Pseudomonas spp.* Total mesophilic counts	Chicken liver	LDPE/PA films. Thyme oil (0.1 and 0.3% (v/w)) was massaged into the product	0.3% thymol EO produced the lowest mesophilic and *Pseudomonas spp.* counts. *B. thermosphacta* increased during storage time under all treatments. Lactic acid bacteria and *Enterobacteriaceae* showed significantly differences between treated and untreated samples.	(156)
	Thymus vulgaris	*Brochothrix thermosphacta* *Escherichia coli*	-	Hake protein edible film – 0.25 mL/g protein	Films containing EO showed to be effective against both *Listeria* strains in agar diffusion method, while	(90)

		Listeria innocua *Listeria monocytogenes* *Pseudomonas putida* *Salmonella typhimurium* *Shewanella putrefaciens*			macrodilution method revealed inhibitory activity for *B. thermosphacta*, *L. innocua*, *S. typhimurium* and *S. putrefaciens.*	
		Listeria monocytogenes	-	Acai edible film – 3 and 6% EO w/w	The highest inhibition zone was presented by 6% EO acai films.	(157)
		Listeria monocytogenes	-	* - 0.05, 0.075 and 0.1% (v/v)	Although thyme EO at 0.1% showed antibacterial activity, samples with 0.05 and 0.075% demonstrated bacteriostatic effects after 48 h storage at 10 ºC.	(135)
		Lactic acid bacteria *Listeria monocytogenes* Total aerobic counts	Mozzarella cheese	Nylon/EVOH/PE bag with paper sachets containing thyme EO – 1% EO	1% EO sachets showed to be effective against all the strains assayed in cheese inoculated with *L. monocytogenes.*	
		Enterobacteriaceae H_2S-producer bacteria Lactic acid bacteria *Pseudomonas spp.* Psychrotrophic count Total viable counts	Rainbow trout fillets	Quince seeds mucilage films – 1, 1.5 and 2% EO (v/v)	At the end of storage time, samples wrapped with 2% EO film did not exceed the acceptable microbial limit (7 log CFU/g) for total viable counts. Successful inhibition of Psychrotrophic bacteria and *Enterobacteriaceae* was also observed. *Pseudomonas spp.* showed to be the most resistant microorganisms. No substantial effects were observed for Lactic acid and H_2S-producer bacteria.	(149)
		Bacillus cereus *Escherichia coli* *Escherichia coli* O157:H7 *Lactobacillus plantarum* *Listeria monocytogenes* *Pseudomonas aeruginosa* *Salmonella typhimurium* *Shewanella putrefaciens* *Staphylococcus aures* *Vibrio cholerae* *Yersinia enterocolitica*	-	Quince seeds mucilage films – 1, 1.5 and 2% EO (v/v)	After the incorporation of 1% EO, films showed antibacterial activity against all strains assayed. Inhibition was increased with increasing concentration of EO. *P. aeruginosa* showed the highest resistance, while *S. putrefaciens* was the most sensitive to thyme films.	(40)

* Essential oil assay, not film. **Abbreviations:** CFU: Colony-forming Unit; CMC: Carboxymethyl Cellulose; EO: Essential Oil; EVA: Ethylene Vinyl Acetate; EVOH: Ethylene Vinyl Alcohol; G-: Gram negative bacteria; G+: Gram positive bacteria; LAB: Lactic Acid Bacteria; LDPE: Low Density Polyethylene; MC: Methylcellulose; MIC:

Minimum Inhibitory Concentration; PA: Polyamide; PE: Polyethylene; PET: Polyethylene Terephthalate; PCL: Polycaprolactone; PP: Polypropylene; PVHO: Polyvinyl Alcohol; REE: Rhubarb ethanol extract; TAB: Total Aerobic Bacteria; WPI Whey Protein Isolated.

Table 2. Antioxidant activity of films incorporated with essential oils.

EO	Species	Film	Antioxidant Assay-Food Matrix (FM)	Observations	References
Basil	*Ocimum basilicum*	Fish skin gelatine (tilapia) (3.5%) Surfactants: Soy lecithin, Tween-20, Tween-80 as EO 1:1	DPPH TEAC FRAP	Films incorporated with basil EO and any of the surfactants presented the highest DPPH and ABTS radical-scavenging activities compared to other films added with citronella, lemongrass or kaffir lime EOs. With respect to the FRAP assay, the basil-film presented the lowest activity. Films prepared with soy lecithin as surfactant presented the best antioxidant activity.	(158)
		Pullulan films EO 6-30 mg/cm^2	DPPH	Films incorporated with basil EO resulted in a concentration-dependent AA increment. Nevertheless, this activity was much lower than that exhibited by the EO alone.	(131)
		Fish skin gelatine (tilapia) (3.5%) Surfactant: Soy lecithin EO 1:1	DPPH TEAC FRAP	Films incorporated with EOs presented a marked antioxidant activity, although it was much lower than the EOs alone. Films incorporated with basil presented the best antioxidant activity through the DPPH and the ABTS radical-scavenging assays in comparison with films with other EOs from plai and lemon. However, films with the basil EO presented the lowest reducing power compared with the other two films.	(159)
Cinnamon	*Cinnamomum zeylanicum*	Cellulose (3.3%) EO 0.05%, 0.1%, 0.25%	β-carotene-linoleate bleaching broth method DPPH	Cellulose-based films containing CEO presented a positive AA against the oxidative bleaching of β-carotene-linoleate, being this activity lower than presented by films added with clove, and higher than presented by films added with green tea (clove > cinnamon > green tea). Films also presented a positive radical-scavenging activity against DPPH radicals following the same order given before. The activity appeared to be higher than that observed in the first method.	(160)
		Low densisty polyethylene films coated with 112 to 527 nm cinnamon EO nanoparticles (C-NPs)	TBARS FM: chilled pork	A significant increase respect to control group was observed during the storage (15 days). Samples treated with C-NPs presented lower TBARS levels than the control, being the NPs loaded with 527 cinnamon EO the more effective a the end of the storage time.	(137)
	Cinnamomun cassia	Alginate-Polycaprolactone (3%-1%) EO 1%	DPPD	The antiradical activity of films incorporated with CEO increased significantly with respect to the control film, although this activity was the lowest observed compared to films added with oregano or savory. Thus, the increment for AP-CEO-films was only 1.2 times higher than	(82)

				ascorbic acid.	
Citronella	*Cymbopogon nardus*	Fish skin gelatine (tilapia) (3.5%) Surfactants: Soy lecithin, Tween-20, Tween-80 EO 1:1	DPPH TEAC FRAP	Films incorporated with citronella EO and any of the surfactants presented a lower DPPH radical- and ABTS radical-scavenging activity than that incorporated with basil EO, But higher than those added with lemongrass or kaffir lime EOs. With respect to the reducing power, the citronella-film presented the second lower activity before basil-films. Films prepared with soy lecithin as surfactant presented the best antioxidant activity.	(158)
Clove	*Eugenia caryophyllata*	Cellulose (3.3%) EO 0.05%, 0.1%, 0.25%	β-carotene-linoleate bleaching broth method DPPH	Cellulose-based films containing clove EO resulted to have a high AA against the oxidative bleaching of β-carotene-linoleate, followed by films added with cinnamon and green tea (clove > cinnamon > green tea). Clove-films also presented the best radical-scavenging activity against DPPH radicals.	(160)
		Sunflower proteins (5%) EO 0.75:1	ABTS FRAP Photochemiluminisce nce assay	Clove EO improved both the radical scavenging and the reducing capacities of films. Moreover, the radical-scavenging ability was much higher against the ABTS radical than against the superoxide anion radical. These results were corroborated with the PCL assay.	(43)
		Hake proteins (cape hake) EO 0.001:4	DPPH Reducing power	The DPPH radical-scavenging activity of films incorporated with clove EO was lower than that presented by the free EO, although it increased the antioxidant ability of the film. This also occurred when the reducing power of the film was studied and compared with the free EO (clove EO > clove-film). In both studies, clove EO resulted more effective than oregano EO (clove > oregano).	(91)
		Chicken feet proteins (5%) Surfactants: Tween-20 (0.25%) EO 1%	DPPH ABTS TBARS FM: sliced cheddar cheese	Films incorporated with clove EO presented the highest DPPH and ABTS radical scavenging activities compared to films containing other EOs from coriander and marjoram. Moreover, the ABTS activity was stronger than the DPPH activity in all cases. In addition, TBARS values in cheese were significantly lower respect to control group after 15 days.	(143)
Green tea	*Camellia sinensis*	*Gelidium corneum*-Gelatine (0.75%-5%) GTE 0.5%, 1.4%, 2.8%, 4.2%	TBARS FM: pork loins	Films containing GTE showed a decreased in the TBARS values compared to the control. However, there was a limited reduction in TBARS values during the storage except at the 10^{th} day.	(161)
		Cellulose (3.3%) EO 0.05%, 0.1%, 0.25%	β-carotene-linoleate bleaching broth method	Cellulose-based films containing GTE resulted to have the lowest AA against the oxidative bleaching of β-carotene-linoleate, as well as the lowest DPPH radical-scavenging activity, compared with clove and	(160)

			DPPH	cinnamon EOs.	
		EVOH (5%) GTE 5%	DPPH TEAC	GTE alone presented high DPPH radical- and ABTS radical-scavenging activities, especially dissolved in ethanol instead of water. When incorporated to the film, the antioxidant activity of the resulted increased, although this activity resulted significantly reduced after the film processing, and also was simulant-dependent, being 95% ethanol simulant the one which presented the best antioxidant activity.	(162)
		Agar (1.5%) GTE 50%	Folin-Ciocalteu test FRAP TEAC	Films containing GTE presented antioxidant activity when FRAP and TEAC assays were performed, although this activity was lower than the observed before a digestion.	(78)
		Polyethylene terephtalate GTE 4 films from 0.7 g GTE/m^2 film to 0.3 g GTE/m^2 film	ORAC Free radicals assay	The antioxidant capacity appeared determined by the solubility of the extracts in ethanol and by the content in catechins, presenting the higher antioxidant activity the GTE soluble in ethanol and with the higher content in catechins. The free radicals assay was performed in films containing 2 GTE at 4 different grammages, being the second film the most antioxidant.	(81)
		Agar (1.5%) Agar-gelatine (1%-0.5%) GTE 50%	FRAP TEAC	Agar films with GTE presented increasing values of FRAP and ABTS with time, which indicates an increasing release of antioxidant compounds from the film. Approximately 60% of the antioxidant power was release during the first 15 min of the assays (16 h). The reducing power of the agar-gelatin film was lower compared to agar-films during all the monitored time.	(89)
		Fish skin gelatine (4%) GTE 0.3%, 0.7%	Folin-Ciocalteu test DPPH Reducing power	The phenolic content increased as the increasing concentration of GTE in the film. The antioxidant activity of the gelatine-GTE film was significantly improved compared to gelatine film alone, and augmented with the increasing GTE concentration.	(163)
		Agar, β-cyclodextrin, activated carbon, methyl-cellulose, Arabic gum, sodium alginate (10%, 35%) GTE 50%, 70% 80%	DPPH Free radicals assay	All the prototypes presented excellent antioxidant activities in both assays, increasing with the percentage of GTE.	(164)
		Fish skin gelatine (3%) GTE 0.01 mg/mL, 1 mg/mL, 5 mg/mL	DPPH Reducing power Retardation of pig oil oxidation	The addition of GTE to the film remarkably increased the antioxidant activity of the film. This activity also increased with the concentration of GTE used. In the same way, the reducing power of films incorporated with GTE was much higher than that from the film alone.	(165)

				When POV was evaluated, the addition of GTE to the film induced a retardation of lipid oxidation.	
		EVOH GTE 5% w/w	DPPH	Films containing GTE presented the highest AA when compared with the film alone and films containing oregano EO, being this activity derived from its content in catechins.	(79)
Lemongrass	*Cymbopogon citratus*	Fish skin gelatine (tilapia) (3.5%) Surfactants: Soy lecithin, Tween-20, Tween-80 EO 1:1	DPPH TEAC FRAP	Films incorporated with lemongrass EO and any of the surfactants presented DPPH and ABTS radical-scavenging activities, although it was lower than those from films added with basil or citronella EOs. With respect to the reducing power, lemongrass-films presented the highest activity when films were prepared with Tween-20 or Tween-80. Nevertheless, films prepared with soy lecithin as surfactant presented the best antioxidant activity.	(158)
Oregano	*Origanum vulgare*	Hake proteins (cape hake) EO 0.001:4	DPPH Reducing power	The DPPH radical-scavenging activity of films incorporated with oregano EO was lower than that presented by the free EO, although it increased the antioxidant ability of the film. This also occurred when the reducing power of the film was studied and compared with the free EO. In both studies, oregano EO resulted less effective than clove EO.	(91)
		Bovine hide gelatine (5%) EO 6000 ppm	FRAP DPPH	Films incorporated with oregano EO presented the highest AA through the FRAP and the DPPH assays compared with films incorporated with lavender EO and control films. However, the AA of films was lower than that presented by the EO alone, possibly due to the interactions between the components of the EO and the proteins from the film.	(92)
		EVOH EO 5% w/w	DPPH	Films containing oregano EO presented a lower AA than films containing GTE.	(79)
	Zataria multiflora	CMC (1%) Surfactants: Tween-80 EO 1%, 2%	DPPH	The AA of films incorporated with oregano EO increased with the increment in EO concentration. Thus, the film containing 3% EO presented the highest AA.	(153)
	Corydothymus capitatus	Alginate-Polycaprolactone (3%-1%) EO 1%	DPPD	The antiradical activity of films incorporated with oregano EO increased significantly in respect to the control group. Thus, the increment for AP-OEO-films was 3 times higher than ascorbic acid.	(82)
Rosemary	*Rosmarinus officinalis*	Chitosan (2%) Surfactant: Tween-80 (0.2%) EO 0.5%, 1%, 1.5%	Folin-Ciocalteu test	Total phenol content of chitosan film increased significantly by incorporating rosemary EO.	(166)
		Chitosan-Clay (2%-1%,	Folin-Ciocalteu test	Total phenol content of chitosan-clay films increased significantly by	(167)

		3%, 5%) Surfactant: Tween-80 (0.2%) EO 0.5%, 1%, 1.5%		incorporating rosemary EO. Moreover, the best values were reached using a film formulated with clay 3% and EO 1.5%.	
Thyme	*Thymus vulgaris*	Chitosan (2%) EO 0.2%, 0.4%, 0.6%, 0.8%, 1%, 1.2%	TEAC	Incorporation of 0.2 and 0.4% thyme EO to the chitosan film did not significantly affect the antioxidant activity. However, the addition of 1 and 1.2% of thyme caused a more marked antioxidant activity (4 and 8 times, respectively) than the control film. The antioxidant compounds were released from the film in the first 2 minutes, except from the film with the higher content of thyme EO.	(168)
		Quince seed mucilage (1%) Surfactant: Tween-80 (0.2%) EO 1%, 1.5%, 2%	Folin-Ciocalteu test DPPH	QSM films showed radical-scavenging activity, although films incorporated with thyme EO exhibited a higher activity, directly proportional to the quantity of EO added to the film. The phenolic content of the films presented a linear correlation with the DPPH assay.	(40)
	Thymus kotschyanus	Starch-Chitosan (1.75%-1%) Surfactant: Tween-80 (0.2%) EO 0.5%, 1%, 2%	Folin-Ciocalteu test DPPH	The DPPH radical-scavenging activity of the S-Ch films significantly was increased with increasing thyme EO concentration (0-2%). In the same way, the total phenolic content in the films significantly increased with the EO concentration.	(169)

Abbreviations: DPPH:2,2-diphenyl-1-picryhidrazyl; TEAC: Trolox-equivalent antioxidant capacity; FRAP: ferric reducing antioxidant power; DPPD: n.n´-diphenyl-p-phenylenediamine; ABTS: 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); TBARS: thiobarbituric acid reactive species; ORAC: oxygen radical absorbance capacity; POV: peroxide value

Table 3. Antioxidant activity of films incorporated with the main compounds from essential oils.

Compound	Film	Antioxidant Assay-Food Matrix (FM)	Observations	References
Carvacrol	*Gelidium corneum* Carvacrol 0.4%, 0.6%, 1%	TBARS FM: ham	Ham samples packaged with films containing 0.6% carvacrol showed lower lipid peroxidation than control films, being significative this difference after 9 days of storage.	(170)
	Linear low density polyethylene-Corn-zein (20%) Carvacrol 1.5%, 3%, 5%	DPPH	DPPH radical-scavenging assay showed an exponential increased liberation of carvacrol with storage time, being this activity concentration-dependent.	(171)
	Chitosan (2%) Surfactant: Tween-80 Carvacrol 0.5%, 1%, 1.5%	DPPH TEAC	The antioxidant capacity of films increased at a rate of 4.8, 4.7, and 2.6 from 1.5% to 0.5%. In the TEAC assay, this increasing rate was 4.82, 4.78, and 4.62 from 1.5% to 0.5%.	(172)
	Polypropylene Carvacrol 8%	DPPH	The antioxidant activity of films incorporated with carvacrol resulted to be lower than those added with thymol.	(173)
Catechin	*Gelidium corneum* (1.5%) Catechin 0.05%, 0.1%, 0.15%	TBARS FM: sausages	The TBARS values increased with increasing storage time. However, sausages packed with films containing GTE showed a significant decrease in TBARS values compared to the control, especially after 12 days.	(174)
	PVA-Starch Catechin 167, 500, 667, 1000, 2000 ppm	TBARS FM: soybean oil	The TBARS values of all treated oil samples were less than those for the control. A significant retardation of the lipid peroxidation was observed in films added with 500 or 667 ppm GTE. Films incorporated with 167 ppm GTE did not present any antioxidation pattern.	(175)
	Zein (0.17 g/mL) Catechins 0.75, 1.5, 2.25, 3, 4.5, 6 mg/cm^2 film	TEAC	Catechins-added films resulted to present less antioxiodant activity than films incorporated with gallic acid. Thus, the TEAC values from GA released from 1.5 to 3 mg/cm^2 phenolic containing films were 3.6 and 4.1 fold higher than the TEAC values from catechin.	(176)
	Poly(L-lactic acid) Catechin 17.6 mg/g	DPPH	PLLA films containing catechin showed a higher percentage of scavenging the DPPH than an extract with the same catechin concentration and also than the control film.	(177)
	Chitosan (4%) Catechin 10%, 20%, 30%, 40%	Folin-Ciocalteu test DPPH	After incorporating catechin, the content of polyphenols increased significantly in respect to control film. This content increased with the concentration of catechin used. The radical-scavenging activity of films incorporated with catechin correlates with this content.	(178)
	Gelatin (3%)	TBARS	Stored samples suffered a TBARS increment during the storage.	(179)

	Catechin 0.5%	FM: minced pork	However, sample wrapped with the gelatine-catechin film presented a non significant increment. Nevertheless, the oxidation was not inhibited, possibly due to the low migration rate of catechins to food.	
	Methylcellulose (2%) Catechin 0.5%, 1%, 2%	DPPH TEAC	Films incorporated with catechins resulted to be effective in scavenging free radicals. Films incorporated with 0.5 and 1% catechins presented a better AA for both assays than those incorporated with 2% catechins.	(180)
Epicatechin	Poly(L-lactic acid) Epicatechin 21.38 mg/g	DPPH	PLLA films containing epicatechin showed a higher percentage of scavenging the DPPH than an extract with the same catechin concentration and also than the control film.	(177)
Eugenol	Linear low density polyethylene-Corn-zein (20%) Eugenol 1.5%, 3%, 5%	DPPH TBARS FM: beef patties	DPPH radical-scavenging assay showed an exponential increased liberation of eugenol with storage time, being this activity concentration-dependent. Moreover, TBARS content in patties increased during the storage, although concentrations of 0.3% or 3% eugenol where enough for significantly decreasing this levels in respect to control group. 0.3% eugenol was sufficient to retard lipid oxidation.	(171)
Quercetin	Chitosan (1%) Quercetin	TEAC	The test film showed strong inhibitory capacity to ABTS radicals, although quercetin *per se* presented the highest antioxidant activity. Chitosan films alone also showed radical-scavenging activity.	(181)
	EVOH (29%) Quercetin 5%	DPPH TBARS FM: brined sardines	The radical-scavenging activity of films incorporated with quercetin was proportional to the concentration released in the different simulants, being higher in those lipophilic simulants. Quercetin-films showed a protective role against lipid oxidation, although films incorporated with green tea extract showed a better profile (TBARS).	(162)
Thymol	Linear low density polyethylene-Corn-zein (20%) Thymol 1.5%, 3%, 5%	DPPH	DPPH radical-scavenging assay showed an exponential increased liberation of thymol with storage time, being this activity concentration-dependent.	(171)
	Bovine gelatine (10%) Thymol 1%, 2%, 4%, 8%	TEAC	Thymol-added films showed a concentration-dependent radical-scavenging activity.	(182)
	Polypropylene Thymol 8%	DPPH	The antioxidant activity of films incorporated with thymol resulted to be higher than those added with carvacrol. Moreover, the best results were obtained in isooctane as simulant.	(173)
Carvacrol/Thymol	Polypropylene Carvacrol-Thymol 4%-4%	DPPH	The combined activity of carvacrol and thymol added to films resulted to present some additive effect of both compounds, with similar radical-scavenging activity than compounds separately.	(173)

Abbreviations: TBARS: thiobarbituric acid reactive species; DPPH:2,2-diphenyl-1-picryhidrazyl; TEAC: Trolox-equivalent antioxidant capacity.

Table 4. Cytotoxic effects of the main essential oils intended to be used in food active packaging.

Plant	Main compound	Experimental model	Assays performed	Exposure conditions	Observations	References
Basil *(Ocimum basilicum)* extract		HepG2	MTT	2.5-50 µg/mL 24 h	The extract was not cytotoxic at the conditions assayed	(101)
Basil *(Ocimum basilicum)* extract	Water extract from basil	SH-SY5Y CV1-P	MTT LDH	0.01-2 mg/mL 12, 24 h	Extract from basil only significantly decreased the metabolic activity of SH-SY5Y cells after 12 h of treatment (2 mg/mL). Similarly, cell death was only observed at the highest concentration assayed	(114)
Basil *(Ocimum basilicum)* leaf extract	EO from leaf and leaf extract	AsPC-1 MiaPaCa	MTT	Ethanolic extract: 0.8 and 80 µg/mL EO: 0.001% and 0.1% v/v 24, 48, 72, 96, 120, 144,168 h	The highest dose of both treatments significantly inhibited the proliferation of cells over time. Higher effects were observed after exposure to the extract in comparison to the EO in both cells	(118)
Cinnamon *(Cinnamomum cassia)* extract	Water soluble cinnamon extract	B16F10, Clone M3, HeLa, Caco-2	EZ-Cytox cell viability assay kit	0.5 mg/mL 48, 72 h	Cinnamon extract strongly inhibited tumor cell proliferation in vitro	(183)
Citronella (Cymbopogon winterianus) EO		Human lymphocytes	TBET MTT	100-2000 µg/mL 3 h	Cell viability showed a concentration-dependent decrease when they were exposed to the EO, being significant at the highest concentrations assayed (1500 and 2000 µg/mL)	(111)
Coriander (*Coriandrum sativum*) leaf extract	Linalool (75.4%), camphor (5.1%), γ – terpinene (4.9%), α-pinene (4.8%), geranyl-acetate (3%), geraniol (2.8%), limonene (2.7%)	293Q WRL-68	MTT	0.4-8 µg/mL 24 h	Whereas low concentration of the extract (0.4 and 0.8 µg/mL) induced cell proliferation, higher concentrations (3.2 to 8 µg/mL) reduced cell survival by 60% or more	(184)

Coriander (*Coriandrum sativum*) extract		RAW 264.7	MTT	100 µg/ml with either the ethyl acetate, chloroform, or methanol fractions of coriander 48 h	The cytotoxicity of the extracts was different with the ethyl acetate fraction being the most toxic (27.9 % cell viability) and the chloroform fraction the less (47.1% of living cells)	(185)
Garlic oil	Diallyl disulfide (56.6%), methyl allyl disulfide (12.2%), methyl allyl trisulfide (12.0%), diallyl trisulfide (11.8%)	HL-60	TBET LDH leakage	0-100 µg/mL 96 h	Garlic oil inhibited cell proliferation in a concentration-dependent way. However, no cytotoxic effect was observed in the LDH leakage assay	(186)
Garlic extracts of two regions of Italy	Water garlic extracts	HepG2	TBET	0.1 , 0.5 and 1% 12, 24, 36, 48 h	Water garlic extracts showed more inhibitory power in the growth of cells than the oil-soluble isolated compound diallyl disulfide	(99)
Garlic extract	Commercially purchase	NCI-H460 NCI-H1299	MTT	0-200 µg/mL 1 h	The growth of both cells was slightly inhibited	(121)
Green Tea (*Camellia sinensis)* extract	Epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin 3-gallate (EGCG)	3T3-L1	MTT	2, 4, 8 % of extract in film 24 h	Only the exposure to the film with 2% resulted in cell viabilities over 50%	(89)
Green Tea (*Camellia sinensis)* extract	EC, ECG, EGC, EGCG and theaflavin	HepG2	TBET MTT	2-80 µg/mL 24, 48, 72, 96 h	A concentration and time-dependent decrease in cell proliferation was observed. Significant reduction in metabolic activity was observed in cells treated with 10 µg/mL and above	(107)
Lemongrass *(Cymbopogon citratus*) EO		Human lymphocytes	TBET MTT	100-2000 µg/mL 3 h	Cell viability showed a concentration-dependent decrease when they were exposed to the EO, being this reduction significant at the highest concentrations assayed (1500 and 2000 µg/mL)	(111)

Marjoran (*Majorana hortensis*) extract	RA (7.87%), luteolin-7-O-glucuronide (3.95%), caffeic acid (1.12%)	MCF-7/Adr MCF-7	MTT	8-2000 mg/L of dried aqueous extracts 48 h	The dried aqueous extract of marjoram showed at low concentrations stimulatory impact on the viability of the wild-type MCF-7 cells, increasing the percentage of living g cells in 20-50%. The calculated EC50 was 220 mg/L	(187)
Onion oil	Dipropyl disulfide (42.1%), dipropyl trisulfide (30.8%), methyl propyl trisulfide (9.9%), methyl propyl disulfide (5.9%)	HL-60	TBET LDH leakage	0-100 µg/mL 96 h	Onion oil inhibited this proliferation in a concentration-dependent way. However, LDH leakage from the cells was not significantly influenced	(186)
Onion (*Allium cepa*) peel extract	p-Coumaric acid (583.2 ± 9.4 µg/g), vanillic acid (245.0 ± 3.5 µg/g), epicatechin (275.0 ± 3.3 µg/g), morin (158.7 ± 5.7 µg/g)	HT-29	MTT LDH leakage	0-250 µg/mL 24 h	The cell viability decreased in a dose-dependent manner	(113)
Origanum majorana extracts		Jurkat	LDH release WST-1	0-8 mg/mL 48, 96 h	The oregano extract decreased the viability of cells in a dose-dependent manner. IC50 of approximately 8 and 5mg/mL after 48 and 96 h, respectively	(119)
Origanum onites EO	Linalool (50.53%), carvacrol (24.52%), thymol (15.66%)	HepG2	CellTiter-Blue® Cell Viability	0-200 µg/mL 24 h	Cell viability decreased in a concentration-dependent way. The EO was found to be less cytototoxic (IC50: 149.12 µg/mL) than carvacrol and thymol (IC50:53.09 and 60.01 µg/mL, respectively)	(104)
Origanum onites EO	Carvacrol (64.3%), linalool (13.8%), ρ-cymene (7.1%), γ-terpinene (3.5%), thymol (1.4%)	5RP7 RATE	MTT	0-500 µg/mL 24, 48, 72, 96 h	A concentration and time-dependent decrease on cell viability at 125, 250 and 500 µg/ml concentrations in both cell lines was observed. In addition, the results indicated 5RP7 cells were more sensitive to this EO than RATECs.	(188)

Origanum vulgare extract	RA (31.8% of identified phenol), ρ-couraaric acid (0.81% of identified phenol), carvacrol (0.75% of identified phenol)	Caco-2	MTT	- 300/500 µg/mL oregano extract - Mix: a mixture of gallic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric, RA, carvacrol Oregano: 12, 24, 48, 72 h / Mix: 24 h	Oregano extract exerted cytotoxic effects on cancer cells in a concentration- and time-dependent manner. However, the mixture of the main compounds present in the extract induced significant toxic effects only at the highest concentration induced	(103)
Palmarosa (Cymbopogon martini) EO		Human lymphocytes	TBET MTT	100-2000 µg/mL 3 h	Cell viability showed a concentration-dependent decrease, being this reduction significant at the highest concentrations assayed (1500 and 2000 µg/mL)	(111)
Rosemary (*Rosmarinus officinalis*) extracts	Two extracts: - AquaROX® 15: RA (17%) - VivOX® 40: carnosic acid (50.27%) and carnosol (5.65%)	HepG2	MTT	0.5-100 µg/mL 21 h	0.5-100µg/ml of AquaROX® 15 extract had no effect on cell viability, while VivOX® 40 extract at concentrations 25 µg/ml and higher (25-100 µg/ml), significantly decreased cell viability	(106)
Rosemary (*Rosmarinus officinalis.*) extract	RA ,carnosol, carnosic acid	A2780	MTT	1/400 - 1/1200 dilution of rosemary ethanol extract 48 h	The antiproliferation activity was influenced by the treatment of the EO extract. The filtrate following ultrafiltration centrifugation reduced proliferation while dialysis treatment cancelled this effect	(127)
Rosemary (*Rosmarinus officinalis*) extract	Ursolic acid (190.0 mg/g dry extract), oleanolic acid (89.7 mg/g dry extract), betulinic acid (46.9 mg/g dry extract)	RINm5F	MTT	12-100 µg/mL 24 h	Significant decrease in cell viability was observed from 25 µg/mL. The calculated IC50 was 35.6 µg/mL	(189)

Thyme (*Thymus zygis subsp. sylvestris*) EO	Carvacrol (25.0%), thymol (23.8%), geranyl acetate (20.8%), geraniol (19.8), linalool (30.0%)	FSDC	MTT	0.08–0.32 µL/mL 3 and 24 h	Only the highest concentration of EO decreased significantly cell viability after 3 h of exposure, being this reduction extremely significant after 24 h	(190)
Thyme (*Thymus serpyllum*) extract	RA (19.3%), luteolin-7-O-glucuronide (10.1%), lithospermic acid (6.27%), caffeic acid (0.29%)	MCF-7/Adr MCF-7	MTT	8-2000 mg/L of dried aqueous extracts 48 h	Increased in viability of the wild-type MCF-7 cells was observed, enhancing the percentage of living g cells in 10-40%. The calculated EC50 was 399 mg/L	(187)
Thyme (*Thymus serpyllum*) extract	RA (14.72 mg/g dry weight (d.w.), salvanolic acid K isomer (3.10 mg/g d.w.), diglucosyl-apigenin (2.22 mg/g d.w.), luteolin-hexoside (2.04 mg/g d.w.)	HepG2	MTT	0-100 mg/mL 24 h	A concentration-dependent decrease was observed. The calculated IC50 value was 4.3 mg/mL	(108)
Thyme (*Thymus vulgaris*) extract	RA (15.1%), luteolin-7-O-glucuronide (6.64%), caffeic acid (0.43%)	MCF-7/Adr MCF-7	MTT	8-2000 mg/L of dried aqueous extracts 48 h	Stimulation in the viability of the wild-type MCF-7 cells was observed at the lowest concentration, increasing the percentage of living g cells in 10–60%. The calculated EC_{50} was 407 mg/L	(187)
Thyme (*Thymus vulgaris*) EO	Thymol (36.1%), ρ-Cymene (22.1%), γ-Terpinene (10.1%)	Differentiated PC12 cells normal and with downregulated Mgst1 (PC12_M)	TBET	100 and 400 µM 24 h	In PC12_M line a significant decrease in the number of viable cells was detected only after treatment with 400 mM thyme oil	(191)

Cell lines: AsPC-1: Human pancreatic adenocarcinoma cell line; A2780: human ovarian carcinoma cell line; Caco-2: human colorectal adenocarcinoma cell line; Clone M3: mouse melanoma cell line; CV1-P: green monkey kidney fibroblast cell line; FSDC: mouse fetal skin dendritic cell line; HeLa: human cervical epithelial adenocarcinoma cell line; HepG2: human liver hepatocellular carcinoma cell line; HL-60: human promyelocytic leukemia cell line; HT-29: human colon epithelial carcinoma cell line; Jurkat: human lymphoblast acute T cell leukemia cell line; LO2: human fetal normal liver cell line; MCF-7: human breast adenocarcinoma cell line; MCF-7/Adr: adriamycin-resistant human breast adenocarcinoma cell line; MiaPaCa: human epithelial pancreatic carcinoma cell line; NCI-H1299: non small cell lung cancer cell line lacking p53 gene; NCI-H460: non small cell lung cancer cell line; PC12: rat adrenal gland cell line; RATEC: rat adipose tissue endothelial cell line; RAW 264.7: murine monocytic

lineage cell line; RINm5F: rat insulinoma cell line; SH-SY5Y: human neuroblastoma cell line; WRL-68: human fetal liver epithelial cell line; 293Q: human fetal kidney epithelial cell line; 3T3-L1: fibroblast cell line; 5RP7: c-H-ras transformed rat embryonic fibroblasts cell line.

Other abbreviations: d.w.: dry weight; EC: epicatechin; ECG: epicatechin-3-gallate; EO: essential oil; EGC: epigallocatechin; EGCG: epigallocatechin 3-gallate; LDH: lactate dehydrogenase; IC50: median inhibitory concentrations that cause approximately 50% cell death; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRU: neutral red uptake; TBET: Trypan blue exclusion test; WST-1: 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate]; RA: rosmarinic acid.

Table 5. Cytotoxic effects of the main components from essential oils intended to be used in food active packaging.

Main compound	Experimental model	Assays performed	Exposure conditions	Observations	References
Apigenin	HepG2	MTT	0-100 µM 24, 48, 72 h	At concentrations greater than 10 µM, apigenin decreased cell survival in a concentration and time-dependent way	(109)
Carvacrol	HepG2	TPC NRU	0.03-3.12 mM 48 h	Carvacrol inhibited the viability and proliferation of HepG2 cells in a concentration-dependent manner, being the IC50 around 0.2 mM) in the proliferation test	(98)
	A549	Cell number TPC	0-1000 µM 24 h	Exposure to 250, 500 and 1000 µM carvacrol decreased cell number and TPC. Although cell number increased in 100 µM treated plates	(192)
	CO25	MTT	1-150 µg/mL 24, 48, 72 h	Carvacrol inhibited growth of myoblast cells in a concentration-dependent way. IC50 value for CO25 myoblast cells was determined as 60 µg/mL.	(193)
	HepG2 Caco-2	TBET	0-900 µmol 24 h	A concentration-dependent decrease was observed in both cell types	(194)
	IPEC-1	MTT	0-1000 µM 48, 96 h	Carvacrol inhibited the proliferation of purified lymphocytes with an IC50 of 182 ± 67 µM	(195)
	Caco-2	MTT	0.7 and 1.4 µg/mL 24 h	The highest concentration assayed significantly decreased cell viability	(103)
	MDA-MB231	MTT	20,40,80,100 µM 24, 48 h	A dose-dependent decrease in the growth of cells was observed. The IC50 of carvacrol was determined to be 100 µM at 48h	(123)
	5RP7 CO25	TBET MTT	0-0.1 mg/mL 24, 48 h	A time- and dose-dependent cytotoxic effects was recorded in both cells, being the IC50 0.04 mg/mL for 5RP7 cells in both exposure times, and 0.1 mg/mL for 24 h and 0.05 mg/mL for 48 h in CO25 cells	(196)
	HeLa SiHa	MTT LDH release	0-500 µg/mL 48 h	Dose-dependent cytotoxic effect was recorded. The IC50 obtained in MTT and LDH both assays were around 50 µg/mL in both cells lines	(197)
	HepG2	CellTiter-Blue® Cell Viability	0-200 µg/mL 24 h	Cell viability decreased in a concentration-dependent way. The IC50 value was 53.09 µg/mL	(104)
	HepG2 LO2	MTT	0–0.4 mmol/L 24 h	No significant effect on LO2 cell viability was observed. However, after a similar treatment of HepG2 cells, the viability decreased in	(105)

				a concentration-dependent way, with an IC50 around 0.4 mmol/L	
	DBTRG-05MG	WST-1	0–1000 µM 24 h	Carvacrol was cytotoxic to human glioblastoma cells in a concentration-dependent manner	(198)
	Cultured primary rat neurons N2a cells	MTT	0-400 mg/L 24 h	Only the high doses of carvacrol (200 and 400 mg/L) exhibited an highly cytotoxic effect on both cell cultures	(199)
	HepG2	TBET MTT	0–1000 µM 24 h	Carvacrol decreased HepG2 cell viability in a concentration dependent manner, reaching a maximum cytotoxic effect at 500-600 µM	(200)
	Caco-2	TPC NRU MTS	0-2500 µM 24, 48 h	A concentration and time-dependent decrease was observed. The EC50 was set at 460 µM after 24 h	(110)
S(+)-carvone	HepG2	TPC NRU	0.06- 3.21 mM 48 h	Carvone inhibited viability and proliferation of HepG2 cells in a concentration-dependent manner. The IC50 ranged from 0.47 mM in the proliferation test to 0.62 mM in viability test	(98)
Catechin gallate	CAL27 HSG S-G HGF-1	NRU	0-250 µM 24, 48, 72 h	The calculated EC50 values in S-G cells, for the 1, 2 and 3 days of exposure were 127, 67 and 58 µM, respectively. The EC50 for other cells were 62 µM, 90 µM and 132 µM for CAL27, HSG and HGF-1 cells, respectively	(117)
Catechin	S-G	NRU	0-500 µM 72 h	After 3 days of exposure the calculated EC50 value was 500 µM	(117)
Cinnamaldehyde	HepG2	TPC NRU	0.16-0.53 mM 48 h	Cell viability and proliferation of HepG2 cells was inhibited in a concentration-dependent manner, being the IC50 around 0.3 mM in both assays	(98)
Citral	Human lymphocytes	TBET MTT	25-800 µg/mL 3 h	Cell viability showed a concentration-dependent decrease, being significant from 100 µg/mL	(111)
DADS	HCT-15 A549 SK MEL-2	TBET	0-500 µM 48 h	A marked growth inhibition after exposure to 100 µM DADS was observed, being skin cells the most sensitive and lung cells the less sensitive	(96)
	NCI-H460 NCI-H1299	MTT	0-50 µM 1 h	The growth of both cells was significantly inhibited by DADS	(121)

	PC-3	TBET	0-40 μM 24, 48, 72 h	The viability was reduced in a concentration-dependent manner upon 24 h. Cells exposed to the highest concentration underwent significant reductions	(201)
	HepG2	TBET NRU	0-100 μM 4 , 20 h	None of the concentrations assayed was found to affect cell viability	(202)
	HepG2	TBET	0-50 μM 12, 24, 36, 48 h	The oil-soluble isolated compound DADS showed lower inhibitory of the growth of in HepG2 than water garlic extracts	(99)
	U937	FCA	0–100 μM 72 h	Significant cell death was only observed in cells exposed to the highest concentration assayed	(203)
	U937	MTT	0–300 μM 48 h	No effect on cell viability was recorded after 48 h of exposure	(204)
DAS	NCI-H460 NCI-H1299	MTT	0-50 μM 1 h	The growth of both cells was significantly inhibited by DAS	(121)
	PC-3	TBET	0-40 μM 24, 48, 72 h	The viability of PC-3 cells was only reduced significantly after 72 h to 40 μM DAS	(201)
	HepG2	TBET NRU	0-100 μM 4, 20 h	None of the concentrations assayed was found to affect cell viability	(202)
	U937	FCA	0–100 μM 72 h	No effect on cell proliferation was observed in cells exposed to up to 100 μM DAS	(203)
	U937	MTT	0–300 μM 48 h	No effect on cell viability was recorded after 48 h	(204)
	ARO	MTT	0–400 μM 24 h	DAS treatment inhibited the proliferation of ARO cells in a dose-dependent manner	(122)
DATS	PC-3	TBET	0-40 μM 24, 48, 72 h	Cell viability was dramatically reduced from 24 h of exposure at all concentrations assayed	(201)
	MCF-7	MTT	0-50 μM 24 h	DATS-induced cytototoxicity can be attenuated by an inhibitor of c-Jun N-terminal kinase	(205)
Diallyl thiosulfinate	U937	FCA	0–100 μM 72 h	No effect on cell proliferation was observed in cells exposed to up to 100 μM of this compound	(203)
Dimethyl thiosulfinate	U937	FCA	0-100 μM 72 h	Living cells were dramatically reduced from 2-5 μM, with an increase in the dead cells number at 5 μM	(203)
DPDS	HCT-15 A549	TBET	0-500 μM 48 h	Both lung and skin tumor cell lines were not sensitive to the addition of 100 μM DPDS	(96)

	SK MEL-2				
	PC-3	TBET	0-160 µM 24 h	The viability of PC-3 cells was not affected, even at 160 µM concentration after 24 h	(201)
	U937	FCA	0–100 µM, 72 h	No effect on cell proliferation was observed	(203)
	Caco-2	TPC NRU MTS	0-200 µM 24, 48 h	No effect in cell viability was recorded	(206)
DPS	PC-3	TBET	0-160 µM 24, h	The viability of PC-3 cells was not affected	(201)
	Caco-2	TPC NRU MTS	0-200 µM 24, 48 h	No remarkable changes in cell viability was observed at any exposure time and concentrations assayed	(206)
Dipropyl thiosulfinate	U937	FCA	0-100 µM 72 h	Living cells were dramatically reduced from 2 µM	(203)
Epicatechin (EC)	S-G	NRU	0-500 µM 72 h	For a 3-day exposure, the calculated EC50 value was 500 µM EC	(117)
Epicatechingallate (ECG)	S-G	NRU	0-50 µM 72 h	The overall cytotoxicity of ECG was similar to its epimer, catechingallate. For a 3-day exposure of this compound, the calculated EC50 value was 36 µM ECG	(117)
Epigallocatechin (EGC)	S-G	NRU	0-500 µM 72 h	For a 3-day exposure, the calculated EC50 value was 324 µM EGC	(117)
EGCGEGCG	S-G	NRU	0-150 µM 72 h	For a 3-day exposure, the calculated EC50 value was 80 µM EGCG	(117)
	U87 U251 LN229	MTT colony-formation	1-100 µM 48 h	EGCG at concentrations ranging from 1 to 100 µM did not substantially reduce cell survival in MTT assays. In contrast, 100 µM was able to completely prevent colony formation, and the IC50 was slightly below 50 µM	(207)
	A431 SCC13	MTT TBET	10- 60 µg/mL 24, 48, 72 h	EGCG was cytotoxic on skin cancer cells, but is not cytotoxic for normal skin cells. However, TBET evidenced cell death at 60 µg/mL in both cells	(130)
Estragole	V79 CHO	MTT	0-3 mM 24 h	Concentration-dependent decrease in cell viability was observed in both cells	(208)

Eugenol	AA8 EM9	MTT	0-800 µM 3, 24 h	Concentration-dependent decrease in viability was observed in both cells exposed to eugenol. No significant reduction in cell viability was found after 3 h of treatment, although it was reduced below 50% when cells were treated for 24 h with concentrations higher than 500 µM or 1000 µM, for AA8 and EM9 cells, respectively	(209)
Geraniol	Human lymphocytes	TBET MTT	25-800 µg/mL 3 h	Cell viability showed a concentration-dependent decrease	(111)
Limonene	SH-SY5Y	FCA	750 µM 24 h	Reduced cell viability was only observed in cells cotreated with limonene and linalyl acetate	(124)
Linalool	HepG2 NC–NC	MTT	0.065, 0.65, 6.5 and 64.8 µM 20 h	No significant effect on cell viability was observed after all exposures. However, incubation of NC–NC cells with 64.8 µM reduced their viability by about 80%	(210)
	HepG2 MCF7 Hek293 NIH3T3 Caco-2	TBET, MTT NRU	0-4 µM 24 h	HepG2 cells were most sensitive to linalool (100% cell death at 2µM), followed by Caco-2 cells (42% cell death at 2 µM). The viability of the rest of the cells was not affected. A concentration-dependent decrease in HepG2 cells was also observed	(112)
	Kasumi-1 HL-60 Molt-4 Raji, K562 Jurkat NB4 THP1 U937 KG-1 L428	MTT	20-640 µM 48 h	Linalool preferentially inhibited growth of a variety of leukemia cells with wild type p53 while sparing normal hematopoietic cells	(120)
	RAW 264.7	MTT	40, 80, and 120 mg/mL 18 h	Cells viabilities were not affected at the concentrations used	(211)
Linalyl acetate	SH-SY5Y	FCA	375 µM 24 h	No cytotoxic effect was induced at concentrations up to 500 µM	(124)
Lithospermic acid	MCF-7/Adr MCF-7	MTT	0.08 - 10 mM 48 h	Cells' survival was lowered by around 60% at 0.63 mM, and by around 80% at 1.25 mM in the case of normal MCF cells. However, the extent of damage observed for MCF-7/Adr cells was	(187)

				lower	
RA	Caco-2	MTT	30 and 60 µg/mL 24 h	Cell exposed to 30 µg/mL rosmarinic acid for 24 h remained unaltered. However the exposure to 60 µg/mL induced a significant reduced viability	(103)
	A2780	MTT	2.5-10 µg/mL 48 h	No significant antiproliferative effect was observed	(127)
	MCF-7/Adr MCF-7	MTT	0.08 - 10 mM 48 h	A marked toxicity was observed at 1.25 mM, decreasing cell viability by around 86%. Against the wild-type MCF-7 cells, a decrease in the number of living cells by 92% at 1.25 mM was observed	(187)
	B16F10 McCoy	TBET NRU	0.001-1000 µM 24 h	The viability of B16F10 cells was altered at 1000 µM RA. However, it was significantly deleterious for McCoy cells at 100 and 1000 µM, reducing their viability to 70 and 35%, respectively.	(212)
	N2A	MTT LDH release	0-500 µM 12, 24 h	Exposures to 1-250 µM for 24 h did not alter the viability. However, higher concentrations were found to be moderately toxic. Rosmarinic acid prevents H_2O_2-induced cell death and LDH leakage in N2A cells	(213)
Thymol	HepG2	TPC NRU	0.25-2.20 mM 48 h	Cell viability and proliferation decreased in a concentration-dependent manner. The IC50 was around 0.7 mM in both assays	(98)
	HepG2 Caco-2	TBET	0-900 µmol 24 h	A concentration-dependent decrease was observed, being HepG2 more sensitive that Caco-2 cells	(194)
	Primary cultures of cortical neurons	MTT LDH release	0-1 mM 30 min and 24 h	Thymol did not affect cell viability	(214)
	HL-60 PBMC	MTT	0-100 µM 24 h	Dose dependent cytotoxic effects were demonstrated on HL-60 cells after 24 h; although no cytotoxic effect was observed in PBMC cells	(125)
	DBTRG-05MG	WST-1	0–800 µM 24 h	In the presence of 200–600 µM thymol, cell viability decreased in a concentration dependent manner. At a concentration of 800 µM, thymol killed all cells	(126)
	Hep G2	CellTiter-Blue® Cell Viability	0-200 µg/mL 24 h	Cell viability decreased in a concentration-dependent way. The IC50 value was 60.01 µg/mL	(104)
	Caco-2	TPC	0-250 µM	No cytotoxic effect was evidenced	(110)

		NRU MTS	24, 48 h		
	Differentiated PC12 cells normal and with downregulated Mgst1	TBET	100 and 400 µM 24 h	No cytotoxic effects were observed after exposure	(191)

Cell lines: AA8: Chinese hamster ovary epithelial cell line; ARO: human anaplastic thyroid carcinoma cell line; A2780: human ovarian carcinoma cell line; A431: human skin cancer cell line; A-549: human alveolar adenocarcinoma cell line; B16F10: mouse skin melanoma cell line; Caco-2: human colorectal adenocarcinoma cell line; CAL27: human tongue epithelial cell line; CHO: chinese hamster ovary cell line; CO25: N-ras transformed myoblast cell line; DBTRG-05MG: human glioblastoma cell line; EM9 : Chinese hamster ovary cell line; HCT-15: human colon tumor cell line; Hek293: human embryonic kidney cell line; HeLa: human cervical epithelial adenocarcinoma cell line; HepG2: human liver hepatocellular carcinoma cell line; HGF-1: human gingival fibroblasts cell line; HL-60: human promyelocytic leukemia cell line; HSG: human submandibular gland cell line; IPEC-1: porcine small intestine cell line; Jurkat: human lymphoblast acute T cell leukemia cell line; Kasumi-1: peripheral blood of an acute myeloid leukemia; KG-1: human macrophage acute leukemia cell line; K562: human chronic myelogenous leukemia cell line; LN229: human glioblastoma epithelial cell line; LO2: human fetal normal liver cell line; L428: Hodgkin's lymphoma cell line; McCoy: mouse fibroblast cell line; MCF-7: human breast adenocarcinoma cell line; MCF-7/Adr: adriamycin-resistant human breast adenocarcinoma cell line; MDA-MB231: human metastatic breast cancer cell line; Molt-4: human acute lymphoblastic leukemia cell line; NB4: human promyelocytic leukemia cell line; NC-NC: human B lymphoblastoid cell line; NCI-H1299: non small cell lung cancer cell line lacking p53 gene; NCI-H460: non small cell lung cancer cell line; NIH3T3: mouse fibroblast cell line; N2a: mouse neuroblastoma cell line; PBMC: human peripheral blood mononuclear cell line; PC12: rat adrenal gland cell line; PC-3: human prostate cancer cell line; Raji: human Burkitt's lymphoma cell line; RAW 264.7: mouse macrophage cell line; SCC13: human skin normal cell line; S-G: immortalized epithelioid cells; SH-SY5Y: human neuroblastoma cell line; SiHa: human cervical epithelial cancer cell line; SK MEL-2: human melanoma cell line; THP1: human acute monocytic leukemia cell line; U251: human glioblastoma cell line; U87: human glioblastoma epithelial cell line; U937: human leukemic monocyte lymphoma cell line; V79: chinese hamster lung cell line; 5RP7: c-H-ras transformed rat embryonic fibroblasts cell line.

Other abbreviations: DADS: diallyl disulfide; DAS: diallyl sulfide; DATS: diallyl trisulfide; DPDS: dipropyl disulfide; EC: epicatechin; ECG: epicatechin-3-gallate; EGC: epigallocatechin; EGCG: epigallocatechin 3-gallate; FCA: flow cytometry analysis; LDH: lactate dehydrogenase; IC50: median inhibitory concentrations that cause approximately 50% cell death; NRU: neutral red uptake; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt); MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RA: rosmarinic acid; TBET: Trypan blue exclusion test; WST-1: 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate]; TPC: total protein content.

Table 6. Cell death evaluation and morphological changes of the main essential oils intended to be used in food active packaging.

Plant	Main compound	Experimental model	Assays performed	Exposure conditions	Observations	Reference
Basil *(Ocimum basilicum)* leaf extract	EO from leaf and leaf extract	AsPC-1 MiaPaCa	Annexin V-FITC kit Hematoxylin and eosin staining and light microscope visualization	Ethanolic extract: 80 µg/ml EO: 0.1% v/v 48 h	The percentage of apoptotic cells was significantly higher upon treatment with the basil extract and EO. Both exposures significantly inhibited the ability of pancreatic cells to migrate and close a scratch wound	(118)
Cinnamon *(Cinnamomum zeylanicum)* EO	Eugenol (77%)	Caco-2	PI staining and TUNEL assay	0.01, 0.05 % 1, 24 h	The addition of the highest concentration increased the percentage of both necrotic (9.11%) and apoptotic (2.51%) cells after 1 h. After 24 h, cells led to general damage presented by 100% cell death. However, 0.01% of cinnamon oil showed no significant damaging effect even after 24 of exposure	(100)
Cinnamon *(Cinnamomum cassia)* extract	Water soluble cinnamon extract	B16F10 Clone M3, HeLa Caco-2	Annexin V-PE, inmunoblot, gen and protein expression	0.5 mg/mL 12, 24, 36, 48 h	Cinnamon extract induced active cell death of tumor cells by up-regulating pro-apoptotic molecules while inhibiting NFκB and AP1 activity and their target genes such as Bcl-2 and BcL-xL	(183)
Citronella (Cymbopogon winterianus) EO		Human lymphocytes	FCA of annexin V-FITC/PI staining	1000, 1500 and 2000 µg/mL 3 h	Induction of considerable apoptosis (39.62%) was observed	(111)
Clove (*Syzygium aromaticum)* EO	Eugenol (85%)	Caco-2	PI staining and TUNEL assay	0.01, 0.05 % 1, 24 h	The exposure to 0.05% for 1 h induced 18.04% of necrotic cells and 5.71% of apoptotic cells, reaching a 100% of death cells after 24 h. No significant damage was observed after 24 of exposure to 0.01%	(100)

Coriander (*Polygonatum odoratum*) extract	8-methyl-dihydrobenzopyranone (8-methyl-DBP), 8-methoxy-dihydrobenzopyranone (8-methoxy-DBP)	T47D MCF-7	Western blot analysis	Ethylacetate extract: 30:1 (v/v) 8-methyl-DBP: 10–80 µM 8-methoxy-DBP: 10-100 µM 12 h	Vietnamese coriander root extract induced apoptosis and G2/M cell cycle arrest in breast cancer cell lines. 8-methyl-DBP was the bioactive compound	(215)
Coriander (*Coriandrum sativum*) leaf extract	Linalool (75.4%), camphor (5.1%) γ –terpinene (4.9%), α-pinene (4.8%), geranyl-acetate (3%), geraniol (2.8%), limonene (2.7%)	293Q WRL-68	FCA	0-80 µg/mL 24 h	The percentage of apoptotic and necrotic cells are increased significantly from 1.6 to 80 µg/mL in both cells. This concentration-dependent increase ranged from 28-41% in apoptotic cells in WRL-68 cells and 9-22% in necrotic cells for 293Q cells	(184)
Garlic extract		NCI-H460 NCI-H1299	Western and northern blot analysis, and acridine orange staining	100 µg/mL 1 h	Garlic extract treatment did not induce any significant apoptotic cells	(121)
Garlic extracts		HepG2	Annexin V-FITC staining and Western blotting	0.1%, 0.5% and 1% 12 , 24, 48 h	Water garlic extracts induced a p53/p21-dependent cell cycle arrest in G2/M phase and apoptosis	(99)
Green Tea (*Camellia sinensis)* extract	Epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate and theaflavin	HepG2	FCA of annexin V-FITC/PI staining	20-80 µg/mL 72 h	Exposure of cells to 20 µg/ml of the extract induced 93.5% of apoptotic cells and 2.3% of necrotic cells. However, 80 µg/ml induced apoptosis in 78.4% of cell population and 11.2% of necrotic cells	(107)
Lemongrass *(Cymbopogon citratus*) EO	Lemongrass	Human lymphocytes	FCA of annexin V-FITC/PI staining	200, 400 and 800 µg/mL 3 h	Significant increase in early apoptosis cells was found at concentrations of 200 and 800 µg/mL with a total of 39.62% of apoptotic cells	(111)
Origanum majorana extracts		Jurkat	Annexin V-FITC, Western blot and FCA	0-2 mg/mL 24 h	The induction of apoptosis observed is related to an up-regulation of p53 protein levels and down-regulation of Bcl-2α	(119)

Oregano (*Origanum onites)* EO	Carvacrol 64.3%, linalool 13.8%, ρ-cymene 7.1%, γ-terpinene 3.5%, thymol 1.4%	5RP7 RATE	DAPI staining Inverted microscope observation for morphological changes	0-500 µg/mL 12 h	125, 250 and 500 µg/mL of oregano EO markedly induced apoptosis of 5RP7 cells. Inhibitory effect of *Origanum onites* EO on angiogenesis and tube formation of RATEC was observed.	(188)
Oregano (*Origanum vulgare*) EO	Carvacrol (55%)	Caco-2	PI staining and TUNEL assay	0.01, 0.05 % 1, 24 h	Concentration and time-dependent increase in cell death was observed, both apoptotic and necrotic. The highest concentration of EO induced 100% cell death and massive loss of monolayer adhesion ability	(100)
Oregano (*Origanum vulgare)* extract	Rosmarinic acid (RA): 31.8% of phenol, ρ-couraaric acid: 0.81% of phenol, carvacrol: 0.75% of phenol	Caco-2	FCA and Western blot analysis	500 µg/ml extract 24 h	Oregano extract leads to growth arrest and cell death in a dose- and time-dependent manner. Oregano triggered both necrotic and apoptotic cell death	(103)
Palmarosa (Cymbopogon martini) EO		Human lymphocytes	FCA of annexin V-FITC/PI staining	1000, 1500 and 2000 µg/mL 3 h	Only a small fraction of cells were found to undergo apoptosis (10.77% apoptotic cells)	(111)
Rosemary (*Rosmarinus officinalis*) extract	RA, carnosol, carnosic acid	A2780	Human apoptosis antibody array kit	1/600 dilution of rosemary ethanol extract 24 h	The extract downregulated 15 genes out of 35 genes assayed. Two genes that were upregulated were that encoding for cytochrome C and heat shock protein 70	(127)
Rosemary (*Rosmarinus officinalis*) extract	Ursolic acid (190.0 mg/g dry extract (d.e.), oleanolic acid (89.7 mg/g d.e.), betulinic acid (46.9 mg/g d.e.)	RINm5F	ELISA test	25 and 50 µg/mL 24 h	Both concentrations of rosemary extract assayed induced apoptotic cell death in RINm5F	(189)
Thyme (*Thymus vulgaris*) EO	Thymol (24%)	Caco-2	PI staining and TUNEL assay	0.01, 0.05 % 1, 24 h	0.01% of thyme EO increased the percentage of both necrotic (12.95%) and apoptotic (0.73%) cells after 1 h, although at 24 h cells were dramatically death. Lower death effects were observed in the exposure to 0.01% even after 24	(100)

Thyme (*Thymus vulgaris*) EO	Thymol (36.1%), ρ-Cymene (22.1%), γ-Terpinene (10.1%)	Differentiated PC12 cells normal and with downregulated Mgst1 (PC12-M)	Inverted microscope observation	100 and 400 µM 24 h	400 µM led to a cytoplasm condensation, cells shrink and loss of contact with the plate in PC12_M cells	(191)

Cell lines: AsPC-1: human pancreatic adenocarcinoma cell line; A2780: human ovarian carcinoma cell line; Caco-2: human colorectal adenocarcinoma cell line; Clone M3: mouse melanoma cell line; HepG-2: human hepatocellular carcinoma cell line; Jurkat: human T-lymphoblastoid cell line; MCF-7: human breast cancer cell line; MiaPaCa: human epithelial pancreatic carcinoma cell line; NCI-H1299: non small cell lung cancer cell line lacking p53 gene; NCI-H460: non small cell lung cancer cell line; PC12: rat adrenal gland cell line; RATEC: rat adipose tissue endothelial cell line; RINm5F: rat insulinoma cell line; SH-SY5Y: human neuroblastoma cell line; T47D: human ductal breast epithelial tumor cell line; WRL-68: human fetal liver epithelial cell line; 293Q: human fetal kidney epithelial cell line; 5RP7: c-H-ras transformed rat embryonic fibroblasts cell line.

Other abbreviations: DAPI: fluorescence microscopy following staining with 40,6-diamidino-2-phenylindole; d.e.: dry extract; ELISA: enzyme-linked immunosorbent assay; EO: essential oil; FCA: flow cytometric analysis; FITC: fluorescein isothiocyanate; PI: propidium iodide; ROS: reactive oxygen species; RA: rosmarinic acid; TUNEL: terminal deoxynucleotidyl transferasemediated dUTP nick end labeling; 8-methyl-DBP: 8-methyl-dihydrobenzopyranone; 8-methoxy-DBP: 8-methoxy-dihydrobenzopyranone.

Table 7. Cell death evaluation and morphological changes of the main components from essential oils intended to be used in food active packaging.

Main compound	Experimental model	Assays performed	Exposure conditions	Observations	References
Apigenin	HPBL	Fluorescence microscope and Western blot analysis	37.2 µM 60 min	Anti-apoptotic effect was observed. Apigenin treatment prior to γ-irradiation showed a significant decrease in the apoptotic incidence (38.91%) when compared to radiation treatment alone	(216)
	HepG2	ELISA (Enzyme-Linked ImmunoSorbent Assay)	20 µM 1, 3, 6, 12, 24, 48 h	Potential apoptotic effect after 24 h was observed	(109)
Carvacrol	HepG2	DAPI staining Inverted microscope observation	0.6 mM 48 h	Carvacrol caused cell detachment. Their irregular shape with several protrusions suggested an apoptotic fate, which was confirmed by the DAPI staining assay	(98)
	A549	Fluorescence microscope observation	0-1000 µM 24 h	Some apoptotic characteristics as well as morphological changes were observed at 500 and 1000 µM	(192)
	Caco-2	PI staining and TUNEL assay	0.37 and 1.83 mM 1, 24 h	The exposure to 1.83 mM for 1 h induced 23.14% of necrotic cells and 3.94% of apoptotic cells, reaching 100% of cell death at 24h. 0.37mM showed no significant damage effect even after 24 of exposure	(100)
	5RP7 CO25	Acridine orange/ethidium bromide staining, flow cytometric analysis (FCA) and analyses of DNA fragmentation	5RP7 cells: 0.02 and 0.04 mg/mL CO25 cells: 0.005-0.1 mg/mL 8, 24 h	5RP7 cells are more sensitive to carvacrol than CO25 cells since carvacrol induced morhological changes characteristic of apoptosis in both cell types but no DNA laddering nor phosphatidilserine localization was observed in CO25 cells	(196)
	MDA-MB231	Annexin V, mitochondrial membrane potential assay, Western blot and FCA Inverted microscope observation for morphological changes	20,40,80,100 µM 24, 48 h	The induction of apoptosis appears to be mediated by cell cycle arrest at S phase, cytochrome c release, decrease in Bcl2/Bax ratio, increase in caspase activity, and cleavage of PARP and fragmentation of DNA. Carvacrol induced morphological changes such as cell shrinkage, rounding of cells and membrane blebbing which are characteristic feature of apoptosis	(123)

	HeLa SiHa	DNA fragmentation assay	0-500 µg/mL 48 h	Carvacrol induced apoptosis of both cells	(197)
	HepG2	Hoechst 33258 stain and FCA	0–0.4 mmol/L 24 h	Inhibition of HepG2 cell growth by inducing apoptosis	(105)
	DBTRG-05MG	Annexin V/PI staining Inverted microscope observation for morphological changes	0-600 µM 24 h	Carvacrol-induced cell death involved apoptosis and necrosis. This apoptosis might be related to increase in ROS. Morphological changes such as cell shrinkage indicating apoptotic cells and cell swelling for necrosis were observed	(198)
	Caco-2	FCA Light and electron microscope observation	0-2500 µM 24, 48 h	Cell death evidenced in the FCA was confirmed in the ultrastructural study	(110)
Carvone	HepG2	DAPI staining Inverted microscope observation	3.2 mM 48 h	Carvone caused cell detachment, but cells seemed to be still vital. Fragmentation of nuclei was observed, which is typical for condensed apoptotic phenotype	(98)
Catechin gallate	S-G	Nuclear staining with acridine orange and TUNEL assay	225, 250 µM 24 h	CG-induced apoptosis was detected in S-G cells exposed for 24 h to 225 µM CG. Morphological changes, characteristic of apoptosis, were noted, highlighting hypercondensed chromatin in spherical or irregular shapes and blebbing of membrane	(117)
Cinnamaldehyde	HepG2	DAPI staining Inverted microscope observation	0.4 mM 48 h	Cell detachment and morphological changes such as irregular shape with several protrusions suggested apoptosis	(98)
Citral	Human lymphocytes	FCA of annexin V-FITC/PI staining	200, 400 and 800 µg/mL 3 h	Citral induced early and late apoptosis, with a 43.94% increase of apoptotic cells with respect to the control	(111)
DADS	HCT-15 A549 SK MEL-2	Morphological changes and DNA fragmentation	0-500 µM 48 h	Exposure to ≥ 100 µM DADS caused apoptosis, which was confirmed by electron microscopy	(96)
	NCI-H460 NCI-H1299	Western and northern blot analysis, and acridine orange staining	20 µM 1 h	Apoptosis was regulated through p53-dependent or p53-independent related Bax/Bcl-2 dual pathway	(121)
	SH-SY5Y	Annexin V-FITC staining and Western blot analysis	0-50 µM 12, 24 h	Arrest of cell cycle in G2/M phase and commitment to apoptosis through the activation of the mitochondrial pathway was observed	(217)
	PC-3	TUNEL assay and DAPI	0-40 µM	DADS only induced significantly apoptosis at 40 µM	(201)

	DU145	staining	1, 4, 16, 24 h	observed by the TUNEL assay	
	HepG2	Annexin V-FITC staining and Western blot analysis	0-50 µM 12, 24, 48 h	DAS did not produced apoptosis in HepG2 cells	(99)
	HL-60 HepG2	TUNEL assay and FCA	1-5 µM 72 h	DADS did not increase significantly the percentage of apoptosis in the absence of N-nitrosamines	(102)
	NCI-H460 NCI-H1299	Western and northern blot analysis, and acridine orange staining	5 µM 1 h	The results showed an increase in the apoptotic cells. The level of p53 protein in H460 cell was increased, suggesting that apoptosis was regulated through p53-dependent pathway	(121)
DAS	NCI-H460 NCI-H1299	Western blot northern blot analysis, and acridine orange staining	20 µM 1 h	The induction of apoptosis by DAS seemed to be regulated through p53-independent related Bax/Bcl-2 pathway	(121)
	PC-3 DU145	TUNEL assay and DAPI staining	0-40 µM 1, 4, 16, 24 h	No significant apoptosis was induced	(201)
	ARO	FCA and Western blot analysis	0–200 µM 24 h	Induction of apoptosis via mitochondrial pathway	(122)
Dimethyl thiosulfinate	U937 NB4 HL-60 MonoMac-6	FCA, ELISA, gelatin zymogaphy and RT-PCR	0-100 µM 72 h	Inhibited cell proliferation in a dose- and time-dependent manner and this process was neither due to cytotoxicity nor apoptosis	(203)
DATS	PC-3 DU145	TUNEL assay and DAPI staining	0-40 µM 1, 4, 16, 24 h	DATS-induced apoptosis involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2	(201)
	MCF-7	Annexin V/PI staining, TUNEL assay and Western blot analysis	0-50 µM 24 h	Apoptosis is mediated through ROS generation and subsequent activation of c-Jun N-terminal kinase and activator protein-1	(205)
DPDS	HCT-15 A549 SK MEL-2	Morphological changes and DNA fragmentation	0-500 µM 48 h	Any concentration assayed caused apoptosis. Electron microscopy showed no significant changes	(96)
	HL-60 HepG2	TUNEL assay and FCA	1-5 µM 0-200 µM	None of the concentrations selected induced a significant percentage of apoptosis. However, DPDS showed a protective effect against an induced apoptosis situation	(102)
	Caco-2	Light and electron microscope observation	0-200 µM 24, 48 h	No significant damage was detected in comparison to the control group	(206)
Dipropyl sulfide	Caco-2	Light and electron	0-200 µM	No significant morphological changes were observed in	(206)

		microscope observation	24, 48 h	Caco-2 cells exposed to DPS	
Dipropyl thiosulfinate	U937 NB4 HL-60 MonoMac-6	FCA, ELISA, gelatin zymogaphy and RT-PCR	0-100 µM 72 h	Inhibited cell proliferation in a dose- and time-dependent manner and this process was neither due to cytotoxicity nor apoptosis	(203)
Epigallocatechin 3-gallate	U251	PI staining and TUNEL assay	20 and 100 µM 48 h	No increase in apoptotic cells were observed in treatment with EGCG alone; however, in combination to temozolomide apoptotic bodies were observed	(207)
Estragole	V79 CHO	Apoptosis annexin assay	0-2000 µM 8, 12, 24 h	Estragole induces apoptosis, but only at the highest concentration, and after long treatment period	(208)
Eugenol	Caco-2	PI staining and TUNEL assay	0.52 and 2.50 mM 1, 24 h	Eugenol caused a concentration-dependent cell death. The exposure to 2.50 mM for 1 h induced 21.64% of necrotic cells and 0.62% of apoptotic cells. While after 24 h of exposure all cells died. The concentration of 0.52 mM showed no significant damage effect even after 24 of exposure	(100)
	AA8 EM9	Caspases activation, and the TUNEL assay	0-800 µM 24 h	Enhanced activation of caspases was evidenced by fluorimetric analysis and fluorescence microscopy in both cells treated with 750 µM eugenol. The percentage of apoptotic cells was 38.9% and 35.8% in AA8 and EM9 cells, respectively.	(209)
Geraniol	Human lymphocytes	FCA of annexin V-FITC/PI staining	1000, 1500 and 2000 µg/mL 3 h	Only a small fraction of cells were found to undergo apoptosis (7.16%)	(111)
Limonene	SH-SY5Y	FCA with Annexin V/PI staining and microscope observation	750 µM 24 h	No change in cell viability was observed following single exposure to limonene. Although the co-treatment with linalyl acetate increased the percentage of apoptotic and necrotic cells (61.5 ± 4.4 %). These findings were corroborated by the morphological alterations observed	(124)
Linalool	Molt-4	FCA with Annexin V/PI staining	20-640 µM 12, 24, 48 h	Linalool-induced apoptosis of Molt-4 lymphoblastic leukemia cells occurred at 12 h, and peaked at 48 h	(120)
Linalyl acetate	SH-SY5Y	FCA with Annexin V/PI staining	375 µM 24 h	Linalyl acetate was not able to induced cell death. However, the co-treatment with limonene increased the number of apoptotic and necrotic cells. Morphological alterations such as cell shrinkage and cytoskeletal alterations were also observed	(124)

Rosmarinic acid	B16F10 McCoy	Phase-contrast microscopy observation	10 µM 24 h	Melanocytes appeared as flattened cells, with scattered and darkened cytoplasm surrounded by multipli protrusions	(212)
Thymol	HepG2	DAPI staining Inverted microscope observation	0.7 mM 48 h	No signs of apoptosis were induced at any of the concentrations tested. However, cells exhibited a typically necrotic death pattern, with cytoplasmic extrusion and no uptake of vital dye	(98)
	Caco-2	PI staining and TUNEL assay	0.80 and 0.17mM 1, 24 h	The exposure to 0.80 mM for 1 h induced 10.08% of necrotic cells and 0.53% of apoptotic cells. Similar results were obtained after 24 h. Slightly lower effects were recorded in exposure to 0.17 m M	(100)
	HL-60 PBMC	DNA fragmentation assay, Western Blot analysis, caspases assay, and apoptosis inducing factor	0-100 µM 24 h	Thymol produced apoptotic cell death based on genomic DNA fragmentation pattern, involving both caspase dependent and caspase independent pathways	(125)
	DBTRG-05MG	Alexa®Flour 488 annexin V/PI staining	0–800 µM 24 h	Treatment with 200, 400, or 600 µM thymol significantly induced apoptosis in a concentration-dependent manner. At 400 and 600 µM, thymol also induced necrosis.	(126)
	Caco-2	FCA Light and electron microscope observation	0-250 µM 24, 48 h	Although no cell death was observed for thymol at any concentration, ultrastructural changes evidenced cellular damage such as lipid degeneration, mitochondrial damage, nucleolar segregation and apoptosis	(110)
	Differentiated PC12 cells normal and with downregulated Mgst1 (PC12-M)	Inverted microscope observation	100 and 400 µM 24 h	PC12_M cells exhibited higher sensitivity to 400 µM thymol showing enhanced tendency to grow in clusters, and to form multilayered colonies	(191)

Cell lines: AA8: Chinese hamster ovary epithelial cell line; ARO: human thyroid anaplastic carcinoma cell line; A549: human alveolar adenocarcinoma cell line; B16F10: mouse skin melanoma cell line; Caco-2: human colorectal adenocarcinoma cell line; CHO: chinese hamster ovary cell line; CO25: N-ras transformed myoblast cell line; DBTRG-05MG: human glioblastoma cell line; DU145: human prostate cancer cell line; EM9 : Chinese hamster ovary cell line; HCT-15: human colon tumor cell line; HeLa: human cervical epithelial adenocarcinoma cell line; HepG-2: human hepatocellular carcinoma cell line; HL-60: human promyelocytic leukemia cell line; HPBL: human peripheral blood lymphocytes cell line; Jurkat: human T-lymphoblastoid cell line; McCoy: mouse fibroblast cell line; MCF-7: human breast cancer cell line; MDA-MB231: human metastatic breast cancer cell line; Molt-4: human acute lymphoblastic leukemia cell line; MonoMac-6: human monocytic cell line; NB4: mouse neuroblastoma cell line; NCI-H1299: non small cell lung cancer cell line lacking p53 gene; NCI-H460: non small cell lung cancer cell line; PBMC: human peripheral blood mononuclear cell

line; PC12: rat adrenal gland cell line; PC-3: human prostate cancer cell line; S-G: immortalized epithelioid cells; SH-SY5Y: human neuroblastoma cell line; SiHa: human cervical epithelial cancer cell line; SK MEL 28: human melanoma cell line; U251: human glioblastoma cell line; U937: human leukemic monocyte lymphoma cell line; V79: chinese hamster lung cell line; 5RP7: c-H-ras transformed rat embryonic fibroblasts cell line.

Other abbreviations: CG: Catechin gallate; DADS: diallyl disulfide; DAS: diallyl sulfide; DATS: diallyl trisulfide; DAPI: with 40,6-diamidino-2-phenylindole; DPDS: dipropyl disulfide; DPS: dipropyl sulfide; EGCG: epigallocatechin 3-gallate; ELISA: enzyme-linked immunosorbent assay; FCA: flow cytometric analysis; FITC: fluorescein isothiocyanate; PI: propidium iodide; ROS: reactive oxygen species; ROS: reactive oxygen species; TUNEL: terminal deoxynucleotidyl transferasemediated dUTP nick end labeling

CAPÍTULO 16/ CHAPTER 16

Sara Maisanaba, Ana I. Prieto, María Puerto, Daniel Gutiérrez- Praena, Esref Demir, Ricard Marcos, Ana M. Cameán

IN VITRO GENOTOXICITY TESTING OF CARVACROL AND THYMOL USING THE MICRONUCLEUS AND MOUSE LYMPHOMA ASSAY

Mutation Research 784-785, 37-44, ***2015***

Elsevier Editorial System(tm) for Mutation Research - Genetic Toxicology and Environmental Mutagenesis
Manuscript Draft

Manuscript Number: MUTGEN-D-15-00039R1

Title: In vitro genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays

Article Type: Research Paper

Keywords: carvacrol, thymol, micronucleus test, mouse lymphoma assay, food packaging

Corresponding Author: Mrs. Sara Maisanaba,

Corresponding Author's Institution:

First Author: Sara Maisanaba

Order of Authors: Sara Maisanaba; Ana Isabel Prieto; María Puerto; Daniel Gutiérrez-Praena; Esref Demir; Ricard Marcos; Ana María Cameán

Abstract: Currently, antimicrobial additives derived from essential oils (Eos) extracted from plants or spices, such as Origanum vulgare, are used in food packaging. Thymol and carvacrol, the major EO compounds of O. vulgare, have demonstrated their potential use as active additives. These new applications use high concentrations, thereby increasing the concern regarding their toxicological profile and especially their genotoxic risk. The aim of this work was to investigate the potential in vitro genotoxicity of thymol (0-250 μM) and carvacrol (0-2500 μM) at equivalent doses to those used in food packaging. The micronucleus (MN) test and the mouse lymphoma (MLA) assay on L5178Y/Tk+/- mouse lymphoma cells were used. The negative results for thymol with the MN with and without the S9 fraction and also with the MLA assay reinforce the view that this compound is not genotoxic in mammalian cells. However, carvacrol presented slight genotoxic effects, but only in the MN test at the highest concentration assayed (700 μM) and in the absence of metabolic activation. The lack of genotoxic response in the MLA assay after 4 and 24 h of exposure indicates a low genotoxic potential for carvacrol. Alternatively, the general negative findings observed in both assays suggest that the MN results of carvacrol are marginal data without biological relevance. These results can be useful to identify the appropriate concentrations of these substances to be used as additives in food packaging.

Sara Maisanaba Hernández
Area of Toxicology. Faculty of Pharmacy. University of Sevilla
C/Profesor García González 2, 41012 Sevilla, Spain
Tel.: +34-954556762; fax: 34-954556422.
E-mail address: saramh@us.es

Sevilla , 7th May 2015

Dear Editor,

Thank you very much for giving us a second opportunity to send you a revised version of our manuscript entitled: "*In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays" , for its publication in the journal "Mutation Research – Genetic Toxicology and Environmental Mutagenesis". We have taken into account all the suggestions and comments from the reviewers, and we appreciate very much their thorough revision. We recognized that their suggestions and comments have improved the quality of the manuscript, and we hope that this new version would be suitable to be published in your journal.

Looking forward to receiving a positive answer from you.

Yours sincerely,

Sara Maisanaba

Highlights

- Interest of carvacrol and thymol for the development of new active packaging
- Carvacrol induces micronucleus at the highest concentration assayed in L5178Y cells
- MLA assay did not show genotoxic response by any of both compounds

In vitro genotoxicity testing of carvacrol and thymol using the micronucleus and ~~the~~ mouse lymphoma assays

Sara Maisanaba[1*], Ana I. Prieto[1], Maria Puerto[1], Daniel Gutiérrez-Praena[1], Eşref Demir[2], Ricard Marcos[3,4], Ana M. Cameán[1]

[1] Area of Toxicology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain.

[2] Department of Biology, Faculty of Arts and Sciences, Akdeniz University, Anmatalya, Turkey.

[3] Group of Mutagenesis, Department of Genetics and Microbiology, University Autonoma of Barcelona, Cerdanyola del Vallès, Barcelona, Spain.

[4] CIBER Epidemiology and Public Health, Instituto de Salud Carlos III, Spain.

*Corresponding author:

Sara Maisanaba

Area of Toxicology, Faculty of Pharmacy, University of Sevilla, C/Profesor García González n°2, 41012 Sevilla, Spain.

E-mail address: saramh@us.es

Tel: +34 954 556762

Fax: +34 954 556422

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Abstract

Currently, antimicrobial additives derived from essential oils (Eos) extracted from plants or spices, such as *Origanum vulgare*, are used in food packaging. Thymol and carvacrol, the major EO compounds of *O. vulgare*, ~~EO~~ have demonstrated their potential use as active additives. These new applications ~~suppose the~~ use ~~of~~ high concentrations, thereby increasing the concern regarding their toxicological profile~~,~~ and especially their genotoxic risk. The aim of this work was to investigate ~~*in vitro*~~ the potential *in vitro* genotoxicity of thymol (0-250 µM) and carvacrol (0-2500 µM) at equivalent doses to those used in food packaging. The micronucleus ~~test~~ (MN) test and the mouse lymphoma (MLA) assay ~~(MLA)~~ on L5178Y/*Tk*$^{+/-}$ mouse lymphoma cells ~~have been~~were used. The ~~negative~~negative results for thymol with the MN with and without the S9 fraction~~,~~ and also with the MLA assay reinforce the view that this compound is not genotoxic in mammalian cells. However, carvacrol presented slight genotoxic effects, but only in the MN test at the highest concentration assayed (700 µM) and in the absence of metabolic activation. The lack of~~, but no~~ genotoxic response ~~was found~~ in the MLA assay after 4 and 24 h of exposure ~~would. This would~~ indicates a low genotoxic potential~~risk~~ for carvacrol. Alternatively, the general negative findings observed in both assays ~~would~~ suggest that the MN results of carvacrol are ~~just~~ marginal data without biological relevance. These results can be useful ~~should be used~~ to identify the appropriate concentrations of these substances to be used as additives in food packaging.

Keywords: carvacrol, thymol, micronucleus test, mouse lymphoma assay, food packaging

1. Introduction

Essential oils (Eos) and their main components are used as additives in the food industry due to their flavour, antimicrobial, and antioxidant properties [1-4]. ~~Additionally, EOs and their main components have been traditionally used to extend the shelf life of food, due to their antimicrobial/antifungal activity [2–4] or their antioxidant properties [5–7].~~ The use of Eos as food preservatives has been limited because high concentrations are required to reach sufficient activity [8]. Active packaging ~~,~~is a new preservation system where volatile compounds from Eos ~~originates~~create a preservative atmosphere. This~~,~~ is considered a good alternative to the direct incorporation of ~~EOs~~ these oils into food. ~~. This method enables a slow release of the agent onto the food surface, maintaining an adequate concentration of the active compounds to preserve the quality of the aliment and~~ avoiding ~~the~~ undesirable secondary effects ~~produced by the direct addition of high levels of EOs~~ [6-7~~9~~ ~~10~~].

~~Currently,~~ Eos derived from oregano species, including its major components~~,~~ carvacrol and thymol, are currently among ~~one of~~ the most frequently ~~EOs~~ used in active food packaging. At present, t~~is that~~ ~~are those~~ ~~derived~~ ~~from oregano species~~ ~~(*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), green tea (*Camellia sinensis*), cinnamon (*Cinnamum zeylanicum*), and clove (*Eugenia caryophyllata*).~~ The ~~Among them, oregano EO and its major components, carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol), have been~~are ~~already used as food preservatives, and the~~ usefulness ~~possibility to use~~of these compounds as substitutes of the common synthetic antioxidants (butylated hydroxyanisole and butylated hydroxytoluene) is under discussion [8~~11~~]. ~~Since~~Because t~~Thus, carvacrol can be directly incorporated by dissolution, microencapsulation, or melt blending in packaging materials, such as wheat gluten coated papers, apple and tomato based films, polypropylene films, and corn zein laminated linear low density polyethylene [12]. On the other hand, thymol has been incorporated by direct dissolution into soy protein isolate based films to package olive oil [13]. Moreover, both carvacrol and thymol are suitable for their microencapsulated use in~~

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polymer matrices for fresh food preservation [14]. The use of carvacrol and thymol, and Eos in general, for these new applications requires high doses, there isconcentrations; thereby, an increasing concern regarding potential exposure to these compounds is arising. ConsequentlyTherefore, more research is needed to establish effective and safe concentrations of Eos and their components [9-101]5].

It is also needed to assess their cytotoxic and genotoxic potential, identifying possible toxic/mutagenic components, and trying to construct an almost complete profile of the risks/benefits associated with their use [16-17].

At present, no single genotoxicity test is able to detect all relevant genotoxic agents. For this reason, batteries of different genotoxicity assays are usually proposed. In all regulatory genetic toxicity testing schemes, the first test to be performed is the bacterial reverse mutation assay, generally conducted in *Salmonella typhimurium* strains (the Ames test). A positive response in the test leads to the presumption that the chemical can be a potential carcinogen. In addition to the Ames test, chemicals are also tested *in vitro* on mammalian cells for gene mutation (usually the mouse lymphoma assay, MLA, that uses the *Tk* gene as a target) and/or for chromosome aberrations (CA) or micronuclei (MN) induction [18]. In fact, iIn accordance with the Guidelines of the Scientific Committee on Food for Safety Assessment of Substances Used in Food Contact Materials [19], the core set of tests used to construct an almost completea profile of the risks/benefits associated with the use of Eos and their components as food contact materials, ceonsists offnf the following 3 *in vitro* genotoxicity *in vitro*studies: 1) a test of the induction of gene mutations in bacteria; 2) a test for the induction of gene mutations in mammalian cells (preferably the mouse lymphoma *Tk* assay); and 3) a test for the induction of chromosomal mutations (chromosome aberrations, (CA or micronucleus/, MN tests) in mammalian cells [11-13]. It should be indicated that aAmong the different *in vitro* mammalian gene-mutation assays Tthe mouse lymphoma assay (MLA) is the most widely used method among various *in vitro* mammalian gene-mutation assays [14]. SimilarlyIn addition, according

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~~to internationally accepted standards~~, the *in vitro* MN assay is ~~being~~ increasingly used in the evaluation of Eos [15-17] instead of the classical CA assay [18-20].~~.~~

~~With respect to the battery of genotoxicity assays conducted according to internationally accepted standards, the *in vitro* MN assay is being increasingly used in the evaluation of EOs [20-24] instead of the classicalCA assay [25-27]. The MN assay allows a high reliable, rapid, and a broad-spectrum determination of DNA damage at the chromosome level [28], being able to detect aneuploidy. The mouse lymphoma assay (MLA) is the most widely used method among various *in vitro* mammalian gene-mutation assays [29]. This assay uses a mouse lymphoblastoid cell line (L5178Y) and has the thymidine kinase (*Tk*) gene as a target [30]. The MLA is able to detect and quantify genetic alterations affecting the expression of the *Tk* gene, covering a wide range of genetic lesions including point mutations, chromosome aberrations, mitotic recombination, and aneuploidy [31-33].These features make this assay particularly useful to evaluate the ability of toxicants to induce mutational events [30, 33].~~

Regarding ~~to~~ the genotoxic potential of thymol and carvacrol~~,~~, several studies have ~~already~~ been conducted with different results, depending~~according~~ on ~~to~~ the assay system and the range of concentrations used~~tested~~ [21-27~~34-40~~]. In bacterial systems, negative results were obtained for thymol using the Ames test [27~~40~~], whereas for carvacrol, the results wea~~r~~e contradictory [28-29~~41-42~~]. Recent studies from our laboratory found that carvacrol exhibited mutagenic potential, being more active in the presence of a metabolic source [27~~40~~]. When ~~In~~ the ~~comet~~c~~C~~omet assay was used~~,using mammalian cells,~~ conflicting results were~~have been~~ reported. ~~Thus, a~~Although Aydin et al. [21~~34~~] demonstrated that carvacrol and thymol induced DNA damage in human lymphocytes, Horváthová et al. [23~~36~~] concluded that ~~both~~ the compounds were~~are~~ not associated with a DNA-damaging effects on human hepatoma HepG2 and colonic Caco-2 cells.

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Taking ~~all~~ these facts into account, the aim of the present work was to investigate ~~*in vitro*~~ the potential *in vitro* genotoxicity/mutagenicity of carvacrol and thymol at relevant concentrations, according to their possible use in food packaging. ~~The~~ MN and MLA tests ~~Two methods have been used, the micronucleus test (MN) and the mouse lymphoma assay (MLA)~~ using the L5178Y/*Tk*$^{+/-}$ mouse lymphoma cell line ~~have been carried out~~were performed. ~~It must be pointed out that, since~~Because no previous data exist ~~using~~for the selected genotoxicity assays, the obtained data ~~will be~~are relevant to assess or confirm their potential health hazards for humans.

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2. Material and methods

2.1. Chemicals

—Thymol (2-isopropyl–5-methylphenol, 99.5%; CAS No. 89-83-8), carvacrol (5-isopropyl-2-methylphenol, 98%; CAS No. 499-75-2), mitomycin (MMC, CAS No. 50-07-7), cyclophosphamide (CP, CAS No. 6055-19-2), methyl methanesulfonate (MMS, 99% purity; CAS No. 66-27-3), thiazolyl blue tetrazolium bromide (MTT, 99.7% purity; CAS No. 298-93-1), trifluorothymidine (TFT, ≥99% purity; CAS No. 70-00-8), thymidine (CAS No. 4449-43-8), hypoxanthine (99% purity; CAS No. 68- 94-0), methotrexate (CAS No. 59-05-2), glycine (≥99% purity; CAS No. 56-40-6), cythochalasin B (Cyt-B, 98%, CAS No. 14930-96-2), Giemsa stain (CAS No. 51811-82-6), and trypan blue solution 0.4% (CAS No. 72-57-1) were purchased from Sigma–Aldrich (Madrid, Spain). RPMI 1640 medium, horse serum, L-glutamine solution (CAS No. 56-85-9), penicillin/streptomycin solution, sodium pyruvate solution (CAS No. 113-24-6), and amphotericin B solution (CAS No. 1397-89-3) were purchased from Gibco (Biomol, Sevilla, Spain).

2.2. Cells and culture conditions

L5178Y/*Tk*$^{+/-}$ mouse lymphoma cells were used for both assays and were provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France), and maintained since then in the laboratory of the University Autonoma of Barcelona (UAB). Cells were cultured to prepare master stocks, which were maintained in liquid nitrogen at a density of 2 × 10^6 cells/mL in a culture medium containing 10% DMSO. They were confirmed as free of mycoplasma by PCR.

L5178Y/*Tk*$^{+/-}$ cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 g/mL streptomycin, 1 mM sodium pyruvate, and 2.5 g/mL amphotericin B. Cells were routinely diluted at 2 × 10^5 cells/mL each day to prevent overgrowth (more than 10^6 cells/mL). Cell density was determined

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with an automated cell counter (~~Countess® Automated cell Counter,~~ Invitrogen®). Cultures were maintained in a humidified incubator with 5% CO_2 ~~in air~~ at 37°C.

2.3. Test solutions

The concentration ranges of thymol and carvacrol ~~concentrations for the tests~~ whereas selected ~~taking into account the considerations described in Llana-Ruiz-Cabello et al. [1],~~ considering the content of these active compounds ~~to be incorporated~~ into ~~the~~ packaging materials and their possible migration to ~~the~~ food, ~~what~~which represents the ~~(~~worst case scenario of exposure~~)~~ [30]. ~~In this sense, the maximum concentration was calculated considering that the active compounds completely migrate from the active package into the food (worst scenario of exposure).~~

Stock ~~concentrations~~ solutions of thymol (0.5 M) and carvacrol (2.5 M) were prepared in dimethyl sulfoxide (DMSO)~~100.%., to dilute the chemicals.~~ RPMI 1640 medium was used to prepare ~~For~~ the different tested~~exposure~~ solutions, ~~where~~in which the final quantity of DMSO was always less than 0.1%. ~~, concentrations RPMI 1640 medium was used, as a solvent, obtaining a final concentration of DMSO less than 0.1%. Cyclophosphamide, MMC, and MMS were dissolved in distilled water.~~ RPMI 1640 medium served as a negative control, ~~whereas~~ile and MMC (0.0625 µg/mL), ~~cyclophosphamide~~ CP (8 µg/mL), and MMS (10 µg/mL) were used as positive controls ~~in the different assays~~. Each ~~compound stock and working~~ solution~~s~~ was~~ere~~ prepared ~~just~~immediately ~~previously~~ prior to its use.

2.4. Micronucleus test (MN)

This ~~experiment~~ assay was ~~carried out~~performed according to the procedure OECD 487 from the Guideline for the Testing of Chemicals [31~~43~~]. ~~. The test was performed in T25 flasks T(~~two duplicate cultures/concentrations were ~~)~~stablished in T25 flasks in the absence or presence of the S9 metabolic activation system.

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2.4.1. ~~Cell viability for~~EC_{50} determination for the MN test

~~The viability trypan blue exclusion test [44] was performed in order to establish the EC_{50} of~~ L5178Y/*Tk*$^{+/-}$ ~~L5178Y~~ cells were exposed to different concentrations of thymol (0-250 µM) or carvacrol (0-2500 µM), and the viability was assessed using the trypan blue exclusion test [32]~~,~~ ~~in order~~ to establish the half maximal effective concentration (EC_{50}). ~~using this value to select the highest concentration of exposure for the MN assay. C~~Thus, ~~c~~ells were seeded at 2 x 10^5 cells/mL and were ~~treated~~ exposed for 24 h ~~different concentrations of each chemical~~ when the metabolic S9 fraction was not used~~,~~ or for ~~3-6~~ 4 h when this fraction was used.~~.~~

~~The EC_{50} after 24 h of treatment was determined using an automated cell counter (Countess® Automated cell Counter, Invitrogen) following the indications given.~~

2.4.2. Micronucleus ~~Genotoxicity~~ assay

L5178Y/*Tk*$^{+/-}$ ~~L5178Y~~ cells were seeded at a concentration of 2 x 10^5 cells/mL ~~per flask~~ and were incubated for 24 h at 37 °C in a~~,~~ 5% CO_2 atmosphere. Then, culture media were removed from the flask by centrifugation (600 rpm, 6 min)~~,~~ and~~-~~ the cells were treated with five different concentrations of thymol (0-250 µM) or carvacrol (0-700 µM), ~~which were~~ ~~defined~~ selected according~~–~~ to the~~ir~~ EC_{50} (without or with S9) previously obtained~~(Figs. 1A; 1B)~~. ~~The solvent~~ RPMI medium was used as a negative control, ~~wh~~ereas ~~and ile~~MMC 0.0625 µg/mL ~~of MMC~~ (in absence of S9) or ~~and~~CP ~~cyclophosphamide~~8 µg/m L ~~ofcyclophosphamide~~ (in presence of S9) were used as positive controls. The exposure times were 24 h in absence of S9 or 4h in the presence of S9, according to the OECD guidelines [31]. After these periods, cells were exposed to ~~In absence of S9, cell cultures treatment last for 1.5-2.0 normal cell cycles (24 h), after this time treated medium was removed and fresh medium with~~Cyt-B (6 µg/mL) ~~was added during 1.5-2.0 normal cell cycles more~~~~during~~for ~~(~~20 h~~)~~. Then, ~~, Cells were treated during 3-6 h in presence of S9 (50 µL, 1%); later, the treated medium with S9 was removed and fresh medium with Cyt-B (6 µg/mL) was added during 1.5-2.0 normal cell cycles more (20 h).~~

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~~After Cyt-B treatment period,~~ cultures were centrifuged ~~(at~~ 600 rpm~~, for~~ 6 min~~),~~ ~~–~~ the supernatant was removed, and~~–~~ cells were subjected to a hypotonic treatment (~~5 mL~~ KCl 0.051 M, 20 min, room temperature). After ~~that,~~this time, cells were centrifuged and ~~another centrifugation was carried out. Cells were~~ fixed with methanol/acetic acid (3:1)~~. at least 3 times. In the last centrifugation, the supernatant was eliminated and the p~~The resultant pellets were ~~as~~resuspended and dropped on ~~clean~~ microscope slides ~~(2 drops of 20 µL each one)~~. After drying, cells were stained with Giemsa 10% solution for 3-5 min.

All slides were encoded by a person ~~outside~~ unconnected with the study, and the scoring was ~~carried out~~conducted by a ~~unique~~ different person using an Olympus BX61 light microscope at 100X ~~magnification under oil immersion~~. ~~MN~~Micronuclei frequencies were ~~analyzed~~ analysed in at least 2000 binucleated cells per concentration~~. (at least 1000 binucleated cells per culture; two cultures per concentration).~~ In addition, cells were scored to evaluate the ~~cytokinesis-block proliferation index (CBPI) [43] or~~ nuclear division index (NDI) [33~~45~~].

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2.5. Mouse lymphoma thymidine-kinase assay ~~(~~MLA~~)~~

~~The MLA was carried out according to Soriano et al. [32]. Taking into account the cell culture conditions, serum concentration was lowered during the treatment to 5% and raised to 20% when cells were dispensed into microwells-plates.~~

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For a successful outcome of the MLA, L5178Y/*Tk*$^{+/-}$ cells were subjected to a cleansing t

~~*2.5.1. Cell cleansing*~~

~~T~~o ~~prepare working stocks for gene-mutation experiments, cultures were~~ purge~~d an~~~~of~~ excess ~~of~~ possible *Tk*$^{-/-}$ mutants by culturing ~~the~~ cells for 24 h in ~~THMG~~ THMG medium ~~, which contains~~(thymidine ~~(~~9 µg/mL~~;~~ ~~),~~hypoxantine ~~(~~15 µg/mL~~;~~ ~~),~~ methotrexate ~~(~~0.3 µg/mL~~L),~~ ~~and~~ glycine ~~(~~22.5 µg/mL). Then, ~~C~~cells were ~~then~~ transferred to THG medium (THMG without methotrexate) for 2 days. The purged cultures were checked for a low background of *Tk*$^{-/-}$

mutants and were stored in liquid nitrogen. The MLA was carried outperformed according to Soriano et al. [34].

~~In first place, 2.5.2. *Gene mutation assay*~~

~~P~~Preliminary experiments were ~~carried out~~conducted to determine the~~he solubility and~~ cytotoxicity of thymol or ~~both~~ carvacrol ~~and thymol to be tested~~. Cytotoxicity was determined by the relative total growth (RTG) ~~following~~ after a treatment of 4 h ~~treatment~~ with thymol (0-250 µM) or~~and~~ 4 h~~-~~ and 24 h ~~treatments~~ with carvacrol (0-2500 µM), without an external metabolic activation source. ~~The RTG measurement takes into account cell loss after treatment, reduction in growth rate over the expression period, and any reduction in cloning efficiency on the day of the selection mutants [46].~~ The recommended highest concentration was one with an RTG of 20% [35~~47~~].

Each main experiment comprised ~~a~~one negative control (RPMI 1640 medium), a~~one~~ positive control (MMS 1 µg/mL), and six concentrations for each tested compound ~~(at least four are recommended)~~. ~~A total of 10^7 cells per culture were grown in R10 medium (10% horse serum) and placed in a series of sterile centrifuge tubes. Solvent, test chemicals or positive control were added. Cells were incubated with gentle shaking (50 rpm) in an incubator at 37°C for 4 h. After exposure, cultures were washed and diluted to 2×10^5 cells/mL. Cells were transferred to culture flasks, counted daily and sub-cultured to 2×10^5 cells/mL for a period of 48 h to allow expression of induced mutations (*Tk* deficient phenotype). Then, cells were seeded in duplicate plates per culture to calculate viability and mutation frequency (MF) on day two. Cell count data were used, in conjunction with viability data on day two, to generate the RTG values corrected for post-treatment toxicity, which provides an indication of post-treatment toxicity in comparison with that of the vehicle control.~~ RTG values were used to decide on the acceptability of the toxicity at each dose level. Thereby, the ~~range~~ selected ranges for thymol and carvacrol, taking into account the obtained RTG values, were 0-250 µM and 0-1500 µM, respectively ~~and 0-250 µM of carvacrol and thymol, respectively~~ (Tables 5-7).

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Once the exposure concentrations were selected,

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~~*2.5.3. Plating for viability*~~

~~C~~cells were plated ~~counted and the~~at a density of ~~was adjusted to~~ 10^4 cells/mL in ~~. A portion of each culture was removed and diluted up to 10 cells/mL. Each culture was dispensed at 0.2 mL/well on~~ 96-well plates (two replicates ~~plates~~per experimental group) ~~in order~~ to evaluate the viability of cells after an incubation ~~were used for each exposure concentration; two plates for the negative control; two plates for the positive control). The plates were incubated~~ of 12 days at 37 °C~~,~~ and 5% CO_2. After this time, viable colonies ~~of plates~~ were counted. In addition,

~~*2.5.4. Plating for 5-trifluorothymidine (TFT) resistance*~~

~~For mutant analysis,~~ two more replicates per experimental group were exposed to TFT ~~was added to cell cultures (10^4 cells/mL) presenting a final concentration of~~ 4 µg/mL for the muta~~n~~tion analysis. ~~Each TFT-treated culture was dispensed at 0.2 mL/well on 96-well plates (two plates were used for each exposure concentration; two plates for the negative control; two plates for the positive control).~~ Plates were also incubated ~~during~~for 12 days at 37 °C~~,~~ and 5% CO_2. To assist the scoring of TFT mutation colonies, MTT 2.5 mg/mL was added to each well and the plates were incubated for 4 h. After this time, the mutant colonies of each plate ~~and counted for the number of microwells containing colonies~~were counted. ~~To assist the scoring of TFT mutation plates, thiazolyl blue tetrazolium bromide solution (MTT, 25 µL, 2.5 mg/mL) was added to each well, and the plates were incubated for 4 h.~~ Colony size was estimated in a similar manner to that described by Honma et al. [36~~48~~]. ~~A small colony was defined as a colony having a size of ≤ 25% of the well diameter. On the other hand, large colonies are defined as those > 25% of the well diameter.~~

2.6. Statistical analysis

~~The IWTG Mouse Lymphoma Workgroup states that biological relevance should be a major factor in MLA data evaluation, determining whether a response was positive or negative [49]. Therefore, the workgroup recommends a biologically relevant approach to evaluate MLA data, which requires that the induced mutant frequency (IMF) exceeds some value based on the global background mutant frequency (MF). This value is the global evaluation factor (GEF), which is considered to be 126 for the microwell version.~~ The induced mutant frequency (IMF) was determined according to the formula IMF~~=~~ ~~is obtained by~~ MF−SMF~~= IMF~~, where MF is ~~one of~~ the test culture mutant ~~frequencies~~ frequency and SMF is the spontaneous mutant frequency. Positive responses are determined as those ~~which,~~that for any treatment meet or exceed the global evaluation factor (GEF, 126 for the microwell assay) ~~plus vehicle control MF,~~ and also when a positive trend ~~test~~ test is obtained.

The statistical approach ~~was~~ included the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the differences in micronucleated cells, NDI and MF between the control and treated cultures. ~~, in the MN assay the two first, and in the MLA the last one.~~ Results were considered statistically significant when the *P*-value was < 0.05.

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3. Results

3.1. Micronucleus test

The doses to be tested in the MN test were selected according to the obtained EC_{50} (Figure 1). ~~G~~The genotoxicity r~~R~~esults obtained from thymol and carvacrol are shown in Tables 1-4. As indicated, both chemicals were evaluated in the absence and presence of the metabolic activation fraction. Neither the frequency of binucleated cells presenting at least one micronucleus (BNMN) nor the frequency of total micronuclei (data not shown), scored per 2000 binucleated cells, showed ~~any~~ a significant increase with respect to the control values in the case of thymol (Tables 1 and 2). ~~However, after treatment with~~When carvacrol was tested~~,~~ a significant ~~but single~~increase of BNMN was observed but only at the highest concentration ~~tested~~ (700 µM) and in the absence of S9 mix. ~~was observed; but~~ No effects were observed ~~not~~ in the presence of ~~the~~ external metabolic activation (Tables 3 and 4). ~~According to the overall data this positive value can be considered as marginal.~~As expected, treatments with ~~the specific positive controls~~ MMC and CP ~~cyclophosphamide, in absence or presence of S9, respectively,~~ induced significant increases in the frequency of BNMN with respect to the~~ir~~ negative controls.

~~On the other hand, t~~Tables 1-4 also show the NDI values obtained after the treatments with thymol and carvacrol. As observed, no significant differences were found in NDI values between the experimental groups and the negative control. Although ~~Moreover,~~ additional nuclear anomalies~~,~~ such as bridges or buds, ~~,~~were checked, ~~but~~ no significant presences ~~have beenwerwase~~ observed (data not shown).

This would indicate that, at the selected doses, no important toxic effects were observed for ~~any of~~ the two tested compounds in the presence of S9~~,~~ ~~and~~or in the absence of S9 ~~also in absence~~ with thymol. Nevertheless, it is important to take into account the positive response of L5178Y/*Tk*$^{+/-}$ ~~L5178Y~~ cells exposed to carvacrol ~~up~~ at 700 µM~~,~~ in the absence of metabolic activation.

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3.2. Mouse lymphoma assay

The results obtained in the MLA after treatments ~~for 4 h~~ of L5178Y/*Tk*$^{+/-}$ cells with thymol and carvacrol for 4 h are shown in T~~t~~ables 5 and 6, respectively. Each compound was tested in two independent experiments, and a good concordance was observed between them. Positive controls with MMS (10 µg/mL) were run in parallel, showing significantly increased mutant frequencies.

~~As observed, t~~Thymol did not present a mutagenic response, and the cytotoxicity test did not show any bioavailability reduction (taking into account the RTG values) after exposure to this compound~~substance~~ (up 250 µM) (Table 5). ~~In relation~~With respect to carvacrol, the obtained results after treatment for 4 h are ~~indicated~~ shown in T~~t~~able 6. ~~As observed, a~~At concentrations higher than 500 µM, pronounced decreases in the relative total growth (RTG) were found. Although higher concentrations (up 2500 µM) were assayed to assess the toxicity, the results are not considered for mutagenicity when the percentage of RTG is lower than 20%, which is the lowest acceptable value. The limit of 20% RTG is widely accepted as the maximum level of cytotoxicity for mutagenicity testing ~~in this case,~~ to avoid biologically irrelevant effects that might occur in heavily stressed cells. However, although cytotoxicity was observed (from 500-1500 µM), no mutagenic response at concentrations up to 1500 µM was obtained. Taking into account ~~what observed in~~the observations of the MN assay, we extended the exposure time with carvacrol ~~until~~ to 24 h. As indicated in T~~t~~able 7, this increased time also ~~reported~~ resulted in non-mutagenic effects. ~~Figure 2 shows the overall results obtained in the MLA assay with thymol and carvacrol.~~

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4. Discussion

Previous studies on the ~~mutagenic and~~ genotoxic~~/mutagenic~~ risk of thymol and carvacrol have ~~already~~ been conducted, but~~although~~ the results we~~are~~ inconclusive ~~but reporting conflictive results~~, especially in the case of carvacrol. ~~[REFS].~~ ~~As consequence~~Therefore, further studies are required to determine the potential ~~real genotoxic~~ risks associated ~~to~~with these ~~that supposes the exposure to these~~ compounds and their use in the food industry. Here, we present studies using the MN and MLA assays to extend our knowledge o~~n~~f the potential genotoxic risk of these compounds.~~.~~

~~In relation to thymol, s~~Several studies of thymol in bacterial systems concluded that it was non~~-~~ ~~e absence of~~ mutagenic ~~potential o~~n~~ver~~at the range of concentrations and conditions tested [28, 37~~41, 50~~]. Specifically, when the ~~regarding to the potential concentrations~~quantities added to food packaging were evaluated,~~,~~ the Ames test revealed ~~no~~ non-mutagenic response in ~~any of~~ the five *S. typhimurium* strains assayed ~~(*S. typhimurium*)~~ in the ~~the~~ presence or absence of S9 [27~~40~~]. In the present study~~our study~~, the range of concentrations selected (0-250 µM thymol) was in agreement with the levels expected in packaging conditions. In this context, the negative results ~~for thymol~~ observed for thymol in the MN assay, with and without the S9 fraction, and also in~~with~~ the MLA assay ~~(without S9)~~, reinforce the ~~view~~ view ~~idea~~that this compound is no~~t~~ genotoxic in~~for~~ ~~in~~ mammalian cells. ~~It should be remarked that in the present study we have used a range of concentrations (0-250 µM) selected according the levels expected in package conditions.~~ This~~is~~ ~~is~~ lack of genotoxicity ~~would~~ confirms ~~those~~ the ~~epreviously~~results previously obtained ~~by~~ using the ~~C~~comet assay in different ~~Caco-2 and HepG2 cells~~cell lines [23, 26-27]. ~~using the comet assay [36, 40]. Similarly, an absence of activity for thymol at biologically relevant concentrations has been reported in V70 Chinese hamster lung fibroblast cells using the comet assay [39].~~On the other hand, although ~~Although~~ a dose-dependent response w~~h~~as ~~been~~ reported in human lymphocytes for thymol using th~~e~~e comet ~~same comet~~ assay, ~~although~~ only the higher concentrations of this compound~~thymol~~ (above 0.1-0.2 mM)

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induced DNA damage~~. No effects were observed at lower concentrations (below 0.1 mM)~~ [21-22~~34 35~~]. These results contrast with those reported by~~In addition~~~~By contrast,~~ Buyukleyla and Rencuzogullari [38] who reported that thymol significantly increased the frequency of sister-chromatid exchanges (SCE)~~,~~ and induced structural CA and MN in human lymphocytes at ~~all the~~ all of the concentrations tested (25-100 µg/mL, equivalent to 17-66.5 µM) ~~in human lymphocytes~~. These ~~differences~~ contradictory results ~~could be~~are related ~~with~~ to the ~~the existing~~ differences between the selected cells types~~lines~~~~experimental model selected~~, ~~being~~with ~~so it seems that~~ human lymphocytes being ~~could be~~ more sensitive than established cell-lines, including ~~compared to~~ ~~to the effects of thymol in comparison to the~~ mouse lymphoma cells (used in our present study). The absence of genotoxicity observed for thymol in the present work could be attributed to its extensively proven antioxidant properties [21, 24, 38]. As a phenolic compound, thymol has high potential to scavenge oxidative radicals by donating protons from ~~their~~its phenolic hydroxyl groups [38]. This fact has already been described in several studies, which reported the antigenotoxic ~~potential~~ or ~~the~~ protective effects of thymol in several experimental models exposed to DNA-damaging agents [21-23, 39]. ~~It is important to point out that n~~No previous genotoxic data for thymol ~~by~~ using the~~from~~ MLA has been reported in the scientific literature ~~so far~~ to compare with our negative results. In this context, ~~it must be remembered that~~ the MLA detects a wide spectrum of genetic damage, including both gene and chromosomal mutations, in contrast with other gene-mutation assays.

~~The absence of genotoxicity of thymol could be attributed to its antioxidant properties demonstrated by several authors [34, 37, 51]. Thymol, as a phenolic compound, has high potential to scavenge radical by its ability to donatea hydrogen atom from their phenolic hydroxyl groups [51]. In fact, several studies reported the antigenotoxic potential or protective effects of thymol in several experimental models against representative DNA-damaging agents, such as H_2O_2 [34, 36], 2-amino-3-methyl-3H-imidazo[4,5-F]quinoline (IQ) and mitomycin C (MMC) [35], and bleomycin [52].~~

In the case of carvacrol, ~~According to the data obtained in~~ the MN assay~~, carvacrol induced~~ showed a~~n~~ slight but significant increase in the frequency of BNMN in the mouse lymphoma cell line, but only at the highest concentration tested (700 µM) and only in the absence of external metabolic activation. However, negative results were obtained ~~in this *in vitro* assay~~ in the presence of the S9 mix. This result ~~would,~~ suggest~~sting~~ a higher genotoxic potential ~~for~~of the parent compound with respect to ~~instead that for~~of its potential generated metabolites. T~~h~~o understand the potential role of metabolic activation on the genotoxicity of carvacrol, we can compare our results on the MN assay with those previously obtained ~~by us esinglelonedamage observed positive results obtained arecould be in agreement with thosee previous mutagenic potentialmutagenic effects exhibited by carvacrol in~~by the Ames test (with and without S9) ~~by us. Surprisingly, i~~In this assay, carvacrol~~carried out in our laboratory, with and without S9, although it~~ displayed a greater genotoxic activity in the presence of the metabolic fraction and at low~~er~~ concentrations (29-460 µM) [27~~40~~]. To solve this ~~type of~~ discrepanc~~ies~~y between assays, ~~Recently, it has been Kirkland et al. [18] have discussed the situation where a chemical present a positive response in the Ames test but a negative one in the MN assay (such as carvacrol with S9). As described by~~ Kirkland et al. [12]~~,~~ proposed that differences between the Ames and the MN assay ~~and indicated that such results need to be considered in~~this discordance could ~~would be~~are explained by assuming~~by~~related light of~~with~~the differences in the metabolism between bacterial and mammalian cells. These authors ~~point outpropose~~proposed that~~. For example:~~ 1) different S9 concentrations are used~~, most usually 10–30% S9 mix~~ in the Ames test~~, but 2–4% S9 mix~~ and in the *in vitro* mammalian cell tests; 2) the duration of exposure is longer in the Ames test (72 h) ~~as~~ compared to ~~the~~ mammalian cell tests (3-6 h in the MN assay); 3) the intrinsic metabolism of the bacterial and mammalian cells may produce different metabolic profiles ~~with a test chemical~~; and 4) the S9 mix ~~used~~ favours oxidative metabolism, which mammalian cells are able to counteract through phase II metabolism to promote detoxification, whereas~~and~~ bacteria may be less able to defend against such oxidative ~~metabolites~~damage ~~whereas~~. ~~Thus, all t~~These points ~~would~~ help ~~us~~ to explain the present data in the MN assay with respect to those previously obtained in the Ames test. Nevertheless, an alternative proposal

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would be to consider the positive result at the concentration of 700 µM ~~just~~ as ~~a~~ marginal, without biological relevance and to attribute non-genotoxic potential to carvacrol in mammalian cells. ~~mammalian cells may have sufficient phase II metabolism to promote detoxification.~~

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~~In spite of this positive effect observed in the MN assay in absence of S9, negative findings were obtained with the MLA assay after 4 h and 24 h of exposure. These differences could be due to the fact that the Ames and chromosome damage (MN, MLA) tests measure different molecular endpoints, and it is possible for a chemical to be capable of inducing base-pair changes, of the type measured by the Ames test, but not induce chromosome breakage or non-disjunction [53] mentioned~~

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The lack of positive effects observed for carvacrol in the MLA assay, both after exposure~~s~~ lasting for 4 and 24 h, would suggest that carvacrol is non-mutagenic, at least under our testing conditions ~~of testing~~. ~~It must be emphasized that~~ MLA detects most of the mutational events associated with the aetiology of cancer and other human diseases, including point mutations and a number of different types of chromosomal mutations. For ~~all these~~ all of these reasons, ~~the~~ MLA is recommended by a number of international regulatory agencies, including the U.S. Food and Drug Administration, the U.S. Environmental Protection Agency, several EU Directives, and the EU REACH integrated testing strategy. Thus, the relevance of the MLA assay ~~would~~ reinforces our suggestion of the low or null genotoxic potential of carvacrol.

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Nevertheless, ~~it must be stated that~~ c~~. In addition, the observed differences could be also explained by the differences that could exist in the uptake and the metabolism of the cells.~~

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~~C~~Contradictory results have been reported in the scientific literature regarding the ~~mutagenicity and genotoxicity~~genotoxicity/mutagenicity of carvacrol. ~~A~~The a~~The us, a~~bsence of mutagenicity ~~activity~~ of this monoterpene has been ~~was~~ observed in the *Salmonella*/microsome reversion test [28~~41~~], whereas ~~and~~ ~~or~~ other studies reported that carvacrol behaves as a strong

antimutagenic agent [2942~~the SCE assay [54], while other studies have reported that carvacrol behaves as . In contrast, according to Ipek et al. [42], carvacrol was reported as a strong direct acting mutagen [42]. ~~]. In mammalian cells, although a ~~Moreover, other~~ in the Ames test system and its metabolites showed weaker mutagenicity at lower concentrations, at least for TA98 strains of *S. typhimurium.* P~~positive ~~genotoxic~~ responses~~ ~~have been~~was obtained in ~~different mammalian cell lines (~~human lymphocytes~~)~~ through the standard c~~C~~omet assay ~~of carvacrol has also been reported by Aydin et al.~~ [21-22~~34-3~~], ~~whereas~~ a lack of genotoxicity using ~~on~~ different~~other~~ cell-lines (Caco-2, HepG2, V79, N2a) has also been reported ~~also showed~~ [26, 40~~5~~].

~~in human lymphocytes using the standard comet assay, whereas a lack of genotoxicity on several human cells, such as HepG2 and Caco-2 cells, was observed [36], as well as in V70 cells [39], fast-growing mouse neuroblastoma N2a cells, and cultured primary rat neurons [55].~~ Confirming the contradictory data on the genotoxic effects of carvacrol, Ündeger et al. [26] revealed non-oxidative DNA damage by low concentrations of this compound using the ~~use of the~~ formamidopyrimidine glycosylase (FPG)-modified ~~comet~~c~~C~~omet assay in V79 cells~~revealed no oxidative DNA damage induction for any of the concentrations assayed [39]~~, whereas~~, but~~ positive effects were recorded ~~were observed~~ in the same assay by using Caco-2 cells exposed to~~after 48 h of exposure at~~ 460 µM carvacrol [27~~40~~]. These contradictory results can be associated ~~to both~~with the pro-oxidant~~s~~ and anti-oxidant~~s~~ properties reported for EOs [10~~16~~]. ~~The increase e increase in the levels of oxidative DNA damage observed for carvacrol in Caco-2 cells is probably a consequence of its capacity to disturb redox cellular equilibrium. In this sense, Llana-Ruiz-Cabello et al. [1] demonstrated~~a significant increase~~s~~ in the levels of reactive oxygen species (ROS) when Caco-2 cells were~~ere exposed to carvacrol for 24 h (460 µM) and 48 h (230 µM). In the case of EOs and their main components, many authors have indicated the involvement of oxidative stress as one of their main toxicity pathways, suggesting a phenolic-like pro-oxidant activity [16].It should be indicated that the majority of antioxidant compounds could have also pro-oxidant activity when their concentrations reach high levels [56]. This has~~

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~~also been reported for major compounds of EOs [57], what would modify their mutagenic and genotoxic properties [58]. This pro-oxidant activity could accelerate the structural changes of membranes (through the oxidation of lipids forming the lipidic bilayers) and, therefore, increase the permeability allowing for an abnormal exchange of substances, including ROS, which could interact with lipids or phenolic components of EOs, increasing the oxidation [16]. As already indicated, there are no previous studies of carvacrol using gene-mutation assays in mammalian cells to compare with our data.].~~

Overall, the present study shows that thymol was no~~t~~ genotoxic when tested with~~in~~ the MN and MLA assays at concentrations ~~potentially~~ intended to be used as additives in food packaging. This fact is reinforced by our previous results in the Ames and c~~C~~comet assays [27~~40~~]. ~~On the other hand, a~~Although carvacrol exhibited~~–~~ a slight genotoxic potential in the MN test, this effect~~s~~ was only observed at the highest concentration assayed (700 µM, equivalent to 10~~,~~.6% of carvacrol in the EO) in L5178Y/*Tk*$^{+/-}$ ~~L5178Y~~ cells, and no genotoxic response was found in the MLA assay after 4 h and 24 h of exposure. This result ~~, what could~~would ~~-~~indicates a low genotoxic risk ~~if already exist~~, or ~~just consider this~~the result ~~as a~~could be considered marginal ~~one~~ and without biological relevance. Nevertheless, this potential discrepancy ~~would~~ suggests ~~to extend~~further studies with carvacrol ~~trying~~ to determine its real genotoxic potential. ~~It must be indicated that the content of many EOs, from the same or from several *Origanum* species, can depend on many factors including cultivation, origin, vegetative stage and growing season of the plants [59]. For example, carvacrol and thymol contents in the same species of oregano (*Origanum onites* from Western Anatolia, Turkey) may change considerably, from 64.3 to 24.52%, and 1.4 to 15.66%, respectively [60-61]. Thus, it is important to select an EO with appropriate proportions of their major compounds, since although we have shown low risk associated to the selected compounds, it would be desirable to choose EOs with high thymol and low carvacrol contents~~variations in their composition have been described [59], ~~for their intended use in food packaging.~~

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5. Conclusion

In the present study, the~~,~~ ~~analysis of the~~ genotoxic potential of thymol and carvacrol ~~has~~ ~~been~~was evaluated ~~by~~ using the MN and MLA tests. ~~by~~ ~~It should be emphasized that t~~This is the first study using the MLA ~~determining~~to determine the potential mutagenic effect of both compounds. ~~R~~The results indicate ~~the MN test revealed~~ that only carvacrol ~~exhibits~~ ~~a~~exhibits a weak genotoxic potential ~~damage~~ on L5178Y/*Tk*$^{+/-}$ ~~L5178Y~~ cells, but only when the MN was used, ~~and~~ at the highest concentration assayed (700 µM), ~~,~~ and in the absence of S9~~. The genotoxic response in the MLA assay was studied for the first time for carvacrol and thymol, obtaining a lack of genotoxicity in both cases.~~. This would suggest a weak genotoxic potential for carvacrol or, alternatively, that these positive findings are ~~just~~ marginal data without biological relevance. Nevertheless, according to the conflict~~ive~~ing data reported for carvacrol~~-~~ in the literature, f~~F~~urther research is needed to confirm ~~their~~ its safety before it is~~they~~ ~~are~~ used as an active additive~~s~~ in food packaging.

Acknowledgements

The authors wish to thank the Spanish Ministry of Science and Innovation (AGL2012-38357-C02-01), co-financed with FEDER funds, and the Junta de Andalucía (AGR-7252) for the financial support ~~fotor~~of this study, and the Biology and Microscopy Services of CITIUS (University of Sevilla)~~,~~ and Gloria Umbert for their technical support.

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Figure Captions

Figure 1. Cell ~~toxicity~~ viability of thymol (A) and carvacrol (B). Treatment lasted for 3-6 h in the presence of an S9 mix and for 24 h in the absence of the S9 mix. Results expressed as the mean ± SD. *Significant differences ~~from~~at *P~~p~~*<0.01.~~and viability was measured by the trypan blue exclusion assay.~~

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~~Figure 2. Mutagenicity of thymol and carvacrol on L5178Y/*Tk*$^{+/-}$ mouse lymphoma cells. Results are from two different experiments.~~

Table Captions

Table 1. Percentage of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured mouse lymphoma cells treated with thymol in the absence of the metabolic fraction S9.

Table 2. Percentage of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured mouse lymphoma cells treated with thymol in the presence of the metabolic fraction S9.

Table 3. Percentage of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured mouse lymphoma cells treated with carvacrol in the absence of the metabolic fraction S9.

Table 4. Percentage of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured mouse lymphoma cells treated with carvacrol in the presence of the metabolic fraction S9.

Table 5. Results of the toxicity and mutagenicity of thymol in mouse lymphoma cells (L5178Y/*Tk*$^{+/-}$) after 4 h of treatment.

Table 6. Results of the toxicity and mutagenicity of carvacrol in mouse lymphoma cells (L5178Y/*Tk*$^{+/-}$) after 4 h of treatment.

Table 7~~:~~. Results of the toxicity and mutagenicity of carvacrol in mouse lymphoma cells (L5178Y/*Tk*$^{+/-}$) after 24 h of treatment.

Table 1

Test substance	Treatment time (h)	Concentrations	BNMN (%)±SE	NDI±SE
Negative control	24	-	0.9±0.1	1.3±0.0
Mitomycin C	24	0.0625 µg/mL	15.6±3.0**	1.4±0.1
Thymol	24	15.62 µM	0.7±0.3	1.4±0.0
	24	31.25 µM	0.6±0.3	1.5±0.1
	24	62.5µM	1.0±0.5	1.5±0.0
	24	125 µM	0.8±0.2	1.3±0.0
	24	250 µM	1.2±0.1	1.5±0.1

Values are expressed as mean ± SD. The significance levels observed are $^{**}P<0.01$ in comparison to the negative control.

Table 2

Test substance	Treatment time (h)	Concentrations	BNMN (%)±SE	NDI±SE
Negative controls	3-6	-	0.9±0.1	2.0±0.1
Cyclophosfamide	3-6	8 µg/mL	3.6±0.4**	2.0±0.0
Thymol	3-6	15.62 µM	0.7±0.3	1.9±0.0
	3-6	31.25 µM	1.0±0.4	2.0±0.1
	3-6	62.5 µM	0.8±0.5	2.0±0.1
	3-6	125 µM	1.1±0.1	2.0±0.1
	3-6	250 µM	1.1±0.1	1.9±0.1

Values are expressed as mean ± SD. The significance levels observed are $^{**}P<0.01$ in comparison to the negative control.

Table 3

Test substance	Treatment time (h)	Concentrations	BNMN (%)±SE	NDI±SE
Negative control	24	-	3.0±1.1	1.5±0.1
Mitomycin C	24	0.0625 µg/mL	31.2±2.2**	1.1±0.0**
Carvacrol	24	43.75 µM	5.0±0.7	1.4±0.0.3
	24	87.5 µM	4.9±0.6	1.4±0.0
	24	175 µM	3.1±0.6	1.4±0.0
	24	350 µM	5.0±0.9	1.5±0.1
	24	700 µM	6.1±1.0**	1.5±0.1

Values are expressed as mean ± SD. The significance levels observed are $^{**}P<0.01$ in comparison to the negative control.

Table 4

Test substance	Treatment time (h)	Concentrations	BNMN (%)±SE	NDI±SE
Negative control	3-6	-	3.1±0.1	1.9±0.0
Cyclophosphamide	3-6	8 µg/mL	5.5±0.8**	1.9±0.1
Carvacrol	3-6	43.75 µM	3.1±0.7	1.9±0.1
	3-6	87.5 µM	3.1±0.4	1.9±0.1
	3-6	175 µM	3.5±0.7	1.9±0.1
	3-6	350 µM	3.3±0.7	1.9±0.0
	3-6	700 µM	3.5±0.3	1.9±0.1

Values are expressed as mean ± SD. The significance levels observed are $^{**}P<0.01$ in comparison to the negative control.

Table 5

Concentration (µM)	Percent plating efficiency	Mutant frequency (x 10^{-6})	Relative total growth	MF (S/L)[a]	IMF (MF-SMF)
Experiment 1					
0	95	138	100	81/57	-
7.8	95	152	110	55/97	14
15.65	80	176	160	68/108	38
31.25	74	177	137	80/97	39
62.5	74	168	140	87/80	30
125	95	182	90	98/84	44
250	104	177	93	81/96	39
MMS (10 µg/mL)	104	773***	87	648/125	635
Experiment 2					
0	100	137	100	77/60	-
7.8	82	162	97	97/65	25
15.65	76	171	118	105/66	34
31.25	75	174	99	108/66	37
62.5	106	110	86	78/32	-27
125	96	146	106	99/47	9
250	76	158	101	103/55	21
MMS (10 µg/mL)	47	986***	77	765/221	849

***$P \leq 0.001$ (significantly different from negative control). [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

Table 6

Concentration (µM)	Percent plating efficiency	Mutant frequency (x 10^{-6})	Relative total growth	MF (S/L)[a]	IMF (MF-SMF)
Experiment 1					
0	78	120	100	69/51	-
62.5	76	112	88	79/33	-8
125	74	141	80	90/51	21
250	93	133	75	77/56	12
500	77	182	74	134/48	62
1000	88	118	69	69/49	-2
1500	78	104	20	60/44	-16
MMS (10 µg/mL)	51	906***	60	805/101	786
Experiment 2					
0	78	132	100	84/48	-
62.5	81	113	99	68/55	-19
125	74	128	102	61/67	-4
250	85	122	89	81/41	-10
500	78	116	73	86/30	-16
1000	75	143	57	73/70	11
1500	56	210	56	126/84	78
MMS (10 µg/mL)	56	604***	61	400/204	472

***$P \leq 0.001$ (significantly different from negative control). [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

Table 7

Concentration (µM)	Percent plating efficiency	Mutant frequency (x 10^{-6})	Relative total growth	MF (S/L)[a]	IMF (MF-SMF)
Experiment 1					
0	110	112	100	75/37	-
62.5	96	111	73	48/63	-1
125	96	118	71	58/60	6
250	115	113	63	67/46	1
500	93	122	58	37/85	10
1000	94	110	54	46/64	-2
1500	113	100	36	32/68	-12
MMS (10 µg/mL)	46	1470***	37	956/515	1358
Experiment 2					
0	82	146	100	76/70	-
62.5	77	191	91	57/134	45
125	94	106	82	31/75	-40
250	65	220	82	103/117	74
500	85	141	55	65/76	-5
1000	80	148	54	46/102	2
1500	77	200	38	76/124	54
MMS (10 µg/mL)	34	2560***	37	1715/845	2414

***$P \leq 0.001$ (significantly different from negative control). [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

Figure(s)

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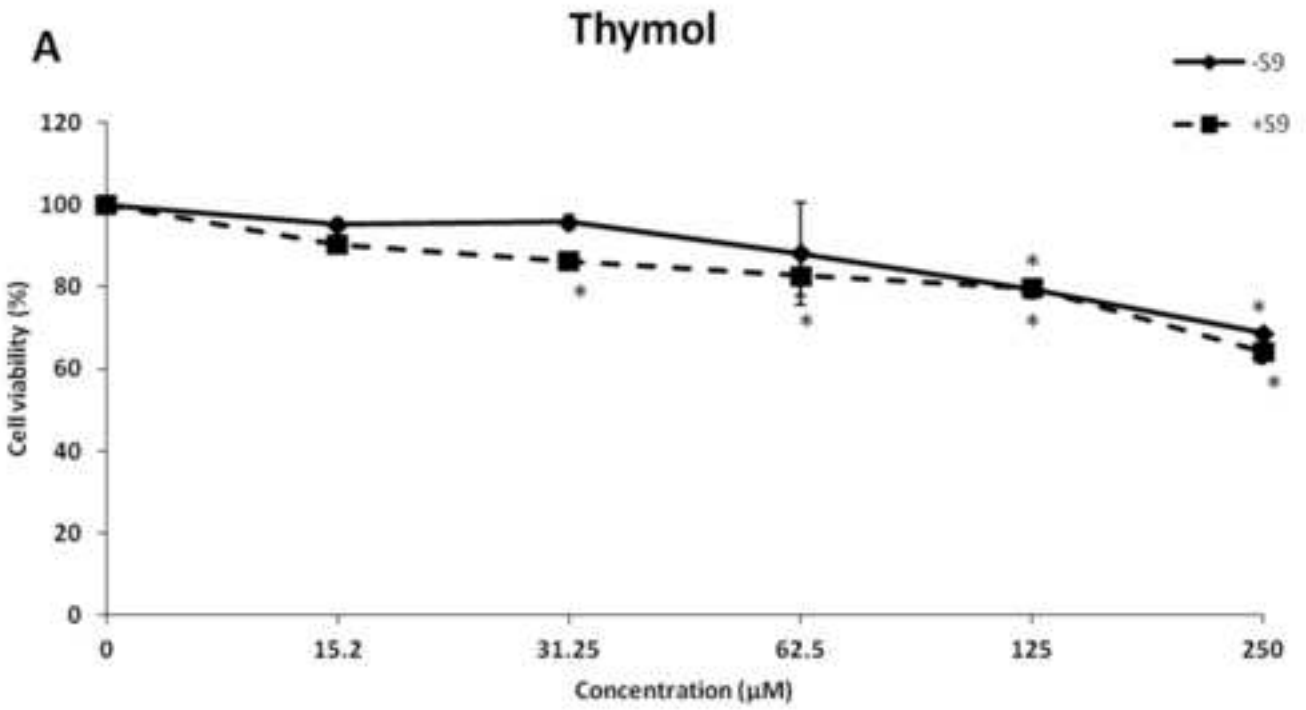

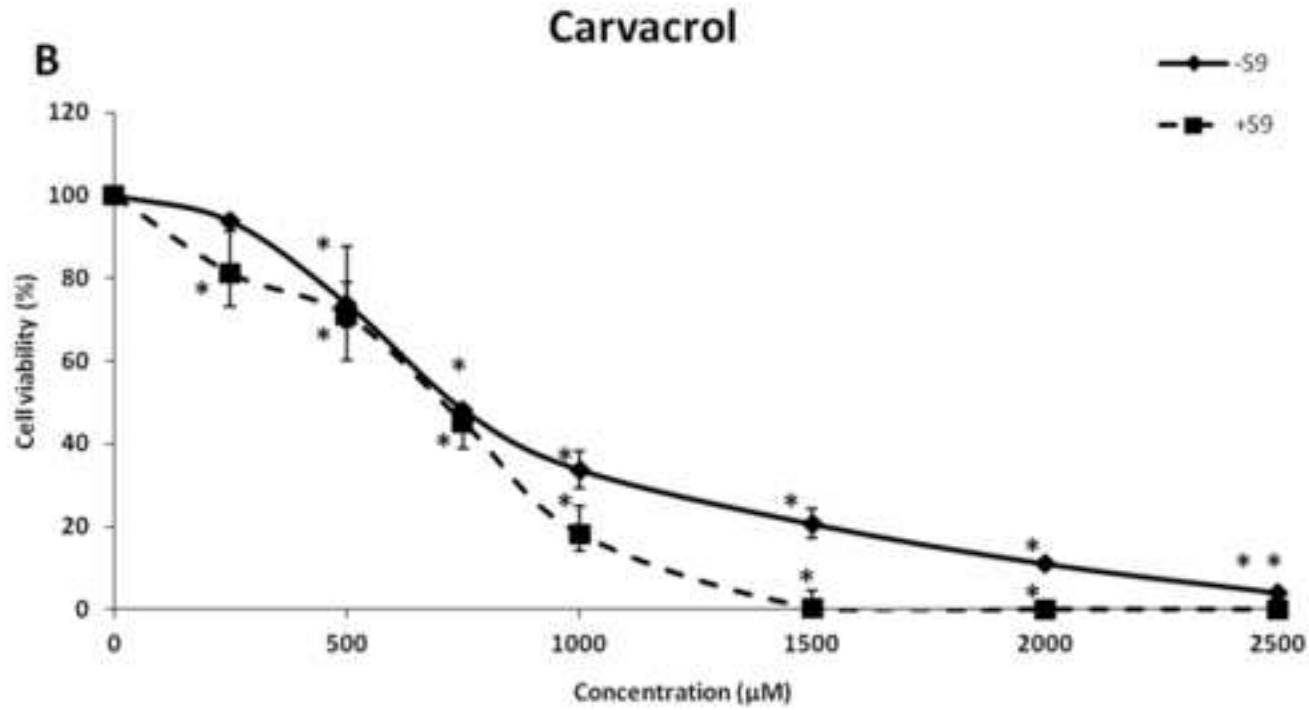

*Conflict of Interest Statement

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Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays	Author name: Sara Maisanaba

Declarations

Mutation Research-Genetic Toxicology and Environmental Mutagenesis requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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The author declares that she has not conflict of interests

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

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The authors wish to thank the Spanish Ministry of Science and Innovation (AGL2012-38357-C02-01) and Junta de Andalucía (AGR-7252) for the financial support for this study.

Signature (a scanned signature is acceptable, but each author must sign)

Print name

SARA MAISANABA

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Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: ***In vitro*** **genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays**	Author name: Ana Isabel Prieto

Declarations

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Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

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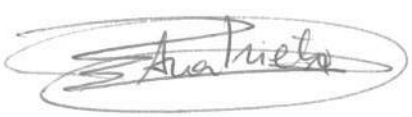
ANA ISABEL PRIETO



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Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays	Author name: María Puerto

Declarations

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The authors wish to thank the Spanish Ministry of Science and Innovation (AGL2012-38357-C02-01) and Junta de Andalucía (AGR-7252) for the financial support for this study.

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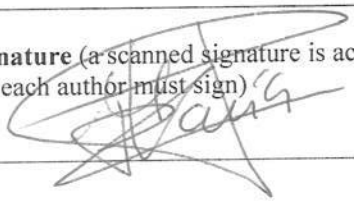

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María Puerto

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Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: ***In vitro*** **genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays**	Author name: Daniel Gutiérrez-Praena

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Article Title: *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays	Author name: Esref Demir

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Print name

Esref Demir

ELSEVIER

Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: ***In vitro*** **genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays**	Author name: Ricard Marcos

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Signature (a scanned signature is acceptable, but each author must sign)

Print name

Ricard MARCOS

ELSEVIER

Mutation Research-Genetic Toxicology and Environmental Mutagenesis

Conflict of Interest Policy

Article Title: ***In vitro*** **genotoxicity testing of carvacrol and thymol using the micronucleus and the mouse lymphoma assays**	Author name: Ana María Cameán

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Signature (a scanned signature is acceptable, but each author must sign)

Ana Mª Cameán

Print name

ANA MARÍA CAMEÁN

V. RESULTADOS Y DISCUSIÓN GENERAL/ RESULTS AND GENERAL DISCUSSION

La apuesta de la industria alimentaria por la incorporación al envasado de materiales de origen natural es hoy una realidad. El objetivo de esta nueva aplicación es aumentar la perdurabilidad de los alimentos perecederos en el mercado, y así, reducir las notables pérdidas económicas que esto supone. Entre los materiales seleccionados para obtener envases con propiedades tecnológicas mejoradas se encuentran las arcillas y organoarcillas derivadas. Por esta razón, decidimos realizar una serie de estudios que nos permitieran conocer, dada la escasez de información hasta el momento, el perfil toxicológico tanto *in vitro* como *in vivo* de cada una serie de arcillas y de los extractos de migración (simulantes alimentarios) obtenidos de materiales nanocompuestos resultantes tras la inclusión de las arcillas en el polímero.

Por otra parte, cabe también destacar el uso de aceites esenciales y compuestos mayoritarios de los mismos con el fin de obtener envases activos, centrados en aprovechar las propiedades antioxidantes/antimicrobianas de las sustancias en cuestión. En este caso se ha abordado la evaluación del perfil genotóxico de timol y carvacrol, dos compuestos mayoritarios del aceite de orégano, siendo necesaria también una exhaustiva evaluación toxicológica antes de la comercialización de los mismos.

1. ESTUDIOS *IN VITRO* SOBRE ARCILLAS Y MATERIALES NANOCOMPUESTOS

Debido al auge del uso de las arcillas en diversas aplicaciones, sobre todo en la industria alimentaria, existen en la bibliografía diversos estudios acerca de la toxicidad inducida por arcillas y materiales nanocompuestos derivados de ellas, principalmente de la montmorillonita (Mt), bentonita y caolinita, en diferentes líneas celulares. Las líneas celulares de origen humano son el modelo *in vitro* escogido con más frecuencia, seguido de líneas celulares de roedores.

En primera instancia, hay diversos trabajos publicados acerca del potencial citotóxico de la Mt, comercialmente conocida como CNa^{+}. En los estudios disponibles en líneas celulares de tejidos diana, como son las pertenecientes a los órganos del sistema digestivo, podemos encontrar una importante disparidad de resultados. Los datos obtenidos en el presente trabajo mostraron de forma general una ausencia de citotoxicidad en las líneas celulares ensayadas (HepG2, Caco-2 y HUVEC) en el rango

de concentraciones seleccionado y con los biomarcadores empleados (captación de rojo neutro (RN), reducción de la sal MTS (MTS) y el contenido proteico total (PT)), observando únicamente leves diferencias en la concentración más alta ensayada (62,5 µg/mL) en HepG2 en el PT tras ambos tiempos de exposición (24 y 48h) y en Caco-2 tras 48h a las dos concentraciones más altas ensayadas (62,5 y 125 µg/mL) en la reducción de la sal MTS. Estos resultados coinciden con los obtenidos por Sharma y col. (2010), los cuales no observaron signos de citotoxicidad en células Caco-2 expuestas a CNa^+ (concentración máxima 226 µg/mL) mediante el ensayo Alarmar Blue. Otros autores también han descrito la ausencia de citotoxidad en la línea celular monocítica U937 expuesta a CNa^+ (Lordan y Higgimbotham, 2012). Sin embargo, existen trabajos publicados en bibliografía que informan del descenso de viabilidad e inhibición en la proliferación celular de células intestinales (INT-407) expuestas a CNa^+ a concentraciones superiores (1000 µg/mL) tras tiempos mayores de exposición (Baek y col., 2012). Por otro lado, Lordan y col. (2011) también observaron efectos citotóxicos en HepG2 tras la exposición a Mt desde concentraciones muy inferiores a las ensayadas en nuestro caso (1 µg/mL) mediante el ensayo de MTT. Otros autores han estudiado el potencial citotóxico en células de ovario de hamster (CHO) expuestas a 1mg/mL de Mt durante 24h, obteniéndose una reducción significativa en la viabilidad celular con respecto al control (Li y col., 2010). Murphy y col. (1993), por su parte, pusieron de manifiesto un descenso de la viabilidad celular en células HUVEC expuestas durante 24h a 0,1 mg / mL bentonitas, aunque se obtuvieron resultados no significativos en las líneas celulares neuronales N1E-115 y ROC-1.

En el caso de arcillas modificadas basadas en CNa^+ se han obtenido una importante diversidad de datos, según el modificador empleado, las concentraciones ensayadas y el modelo experimental seleccionado. Cabe destacar los efectos citotóxicos obtenidos en nuestro caso por C30B, Clay2, Clay4A y Clay4B en las líneas celulares elegidas (HepG2, Caco-2 y HUVEC) mediante los biomarcadores seleccionados (RN, MTS y PT), mientras que no se obtuvieron efectos significativos de descenso de la viabilidad celular con C20A, Clay1 y Clay3. Hay autores que sugieren que el modificador incluido en la arcilla madre interviene en la modulación de la toxicidad ejercida por la arcilla resultante (Sharma y col., 2010).

Los resultados obtenidos con C30B en el presente estudio son avalados por los resultados publicados por Sharma y col. (2010), los cuales observaron una depleción de

la viabilidad de las células Caco-2 expuestas a C30B a la concentración más alta ensayada (226µg/mL). Otras arcillas disponibles comercialmente también han sido evaluadas como es el caso de C93A, la cual dio lugar en células HepG2 a un descenso de la viabilidad celular y a un incremento de la liberación de la lactato deshidrogenasa (LDH) dependiente del rango de concentración ensayado (0-1000µg/mL) (Lordan y col., 2011).

Hasta el momento los estudios toxicológicos llevados a cabo con arcillas modificadas con silanos, como es el caso de Clay3, Clay4A y Clay4B, son muy limitados. Vergaro y col. (2010) evaluaron nanotubos de halosita funcionalizados con aminopropiltrietoxisilano (APTES), modificador presente en Clay3. Los resultados obtenidos en células epiteliales HeLa y MCF- 7 (epiteliales) por exposición a la arcilla funcionalizada (1-1000µg/mL) indicaron una inhibición en el crecimiento de forma dependiente en el tiempo en ambas líneas celulares.

Por otro lado, Verma y col. (2012) estudiaron el efecto de la Mt modificada con oligo(estireno-co-acronitrilo) (0-250 µg/mL) en células renales (HEK293) y fibroblásticas (NIH 3T3), obteniendo un descenso significativo de la viabilidad celular mediante el ensayo de MTT, así como un aumento de la liberación de la LDH. Sin embargo, es muy curioso destacar que la Mt sin modificar presentó mayor toxicidad que la modificada bajo las condiciones ensayadas (Verma y col., 2012). Otros autores han evaluado el potencial citotóxico de la bentonita nativa (arcilla con estructura similar a la Mt) y modificada con H_2SO_4 en células HMy2.CIR durante 4 y 24h, observando una depleción de la viabilidad por exposición a ambas, siendo más significativo en el caso de la arcilla modificada (Meibian y col., 2010).

Una vez realizados los estudios de citotoxicidad con las arcillas disponibles continuamos con la evaluación del perfil toxicológico que presentaba cada una, ahondando en las alteraciones morfológicas celulares producidas tras su exposición, determinación de los mecanismos de acción (incluyendo estrés oxidativo, genotoxicidad/mutagenicidad, inflamación y mecanismos de muerte celular). De este modo se trataba de esclarecer la mayor información posible acerca del perfil toxicológico de arcillas destinadas a aplicaciones en la industria alimentaria.

Entre los marcadores de toxicidad celular más frecuentes usados están los que se basan en la alteración de la morfología celular (Borenfreud y Borrero 1984). Los datos

recogidos en nuestro estudio muestran una alteración de diversas estructuras celulares como mitocondrias, formación de heterofagosomas y gotas lipídicas tras exposición de HepG2 a C30B, y, variaciones en el aparato de Golgi y segregación nuclear tras exposición de Caco-2 a la misma arcilla modificada, durante 24 y 48h en ambos casos. De acuerdo con estos resultados, encontramos en bibliografía un estudio llevado a cabo en células HepG2 expuestas a CNa^+ y C93A en el cual donde se observa muerte celular y degradación de orgánulos tras la exposición a ambas después de 24h de exposición (Lordan y col., 2011). Del mismo modo, Murphy y col. (1993a,b) examinaron cambios morfológicos producidos en HUVEC y cultivos primarios de neuronas expuestas a 0.1 mg/mL de un grupo de arcillas entre la que se encontraba la Mt, además de bentonita, erionita y caolinita, mostrando la Mt un mayor daño morfológico. Únicamente, Geh y col. (2006) informaron sobre una ausencia de alteraciones morfológicas en la línea celular de fibroblastos IMR90 expuesta a bentonitas nativas y modificadas (10 µg/cm^2).

Por otro lado, valoramos la posibilidad de estudiar *in vitro* el estrés oxidativo como posible mecanismo de acción tóxica, mediante la generación de especies reactivas de oxígeno (ERO) y el descenso del contenido de glutatión (GSH). Varios autores han evaluado los efectos relacionados con la generación de ERO y otras alteraciones en diversas líneas celulares expuestas a arcillas no modificadas y modificadas. Respecto a los datos obtenidos acerca de CNa^+ se expone una gran disparidad de los mismos en los trabajos disponibles en la literatura científica, dependiendo sobre todo del modelo experimental seleccionado. En este sentido, Baek y col. (2012) indicaron una producción significativa de ERO a la concentración más alta ensayada de Mt expuesta a la línea celular intestinal INT-407 (1000µg/mL) tras 24h de exposición, aunque a tiempos superiores (48-72h) de ensayo el daño apareció desde concentraciones mucho menores (a partir de 50µg/mL). De forma similar, Lordan y col. (2011) evidenció la generación de ERO en HepG2 expuestas a CNa^+ desde concentraciones muy bajas (50µg/mL) a todos los tiempos de exposición ensayados (4, 16 y 24h). Sin embargo, por su parte, Sharma y col. (2010) publicó la ausencia de alteración por exposición de CNa^+ a Caco-2 (226 µg/mL).

En consideración a las arcillas modificadas con sales de amonio cuaternario, en nuestro estudio sólo se ha observado un incremento en la generación de ERO con un descenso significativo del contenido en GSH en células Caco-2 expuestas a C30B a la concentración más alta ensayada (40 µg/mL), mientras que las células HepG2

únicamente mostraron una depleción del compuesto antioxidante en todas las concentraciones ensayadas (22, 44 y 88 µg/mL) pero no un incremento de ERO. En el caso de la exposición de ambas líneas a Clay2, sólo se observó alteración en el contenido de GSH en la línea hepática, presentándose una disminución en todas las concentraciones ensayadas a ambos tiempos de exposición (22, 44 y 88 µg/mL). En contraste con estos resultados, otros estudios no observaron generación de ERO tras la exposición de C30B a Caco-2 a concentraciones superiores a las ensayadas en nuestro caso (0-226 µg/mL) (Sharma y col., 2010). Sin embargo, C93A expuesta a HepG2 dio lugar a un incremento en la producción de ERO, siendo las concentraciones de exposición más elevadas que las seleccionadas en nuestro estudio (1000µg/mL), dando lugar a una respuesta de estrés posiblemente debido no sólo al efecto tóxico sino también pudiendo estar relacionada con la posible precipitación de la arcilla y generación de estrés físico en las células.

Respecto a las arcillas modificadas con silanos, Clay3, Clay4A y Clay4B, se observaron niveles significativos de ERO en el caso de HepG2 expuestas a Clay4A y Clay4B, y Caco-2 expuesta a Clay4A; sin embargo, no se produjo ninguna alteración por exposición a Clay3. Por otro lado, sólo en el caso de ambas líneas celulares expuestas a Clay4B se obtuvo un descenso en el contenido de GSH a la concentración más alta ensayada para Caco-2 (40µg/mL) y a las dos concentraciones superiores para HepG2 (42,5 y 85µg/mL). Podríamos suponer que el papel que juega el modificador empleado en cada caso cuenta con un papel crucial, modulando la toxicidad generada. Hasta el momento, los estudios disponibles en bibliografía sobre arcillas modificadas con silanos son inexistentes.

Además de la Mt y arcillas modificadas basadas en ella podemos encontrar trabajos sobre otras arcillas con estructura similar. Meibian y col. (2010) determinaron la generación de ERO, la actividad de la superóxido dismutasa (SOD) y peroxidación lipídica (LPO) en una línea celular linfoblastoide expuesta a dos bentonitas, una nativa y otra modificada con H_2SO_4. Los autores observaron que la mayor respuesta de estrés oxidativo fue mostrada con la bentonita modificada bajo las condiciones ensayadas.

Por otra parte, teniendo en consideración la variedad de mecanismos que conducen al daño en el ADN inducido por xenobióticos, y, la gama de acontecimientos genotóxicos y mutagénicos que pueden ocurrir como resultado, se requiere una batería

de ensayos para el establecimiento del perfil y potencial genotóxico de una sustancia que esté siendo investigada (Doak y col., 2012). En la actualidad, los ensayos *in vitro* de genotoxicidad y mutagenicidad se incluyen entre el conjunto básico de pruebas de toxicidad que debe llevarse a cabo para la evaluación de la seguridad, requerida dicha información por las autoridades europeas con el fin de autorizar nuevas sustancias para ser usadas como materiales en contacto con alimentos (EFSA 2011b, 2015). Dentro de los ensayos de genotoxicidad reclamados por la EFSA se encuentran el ensayo de Micronúcleos (MN) y el test de Ames (2011b, 2015). Además, el ensayo Cometa se lleva a cabo en muchas ocasiones con el fin de confirmar resultados no concluyentes, contradictorios o equívocos en los tests anteriores , ya que nos proporciona una información más completa acerca del potencial genotóxico de la sustancia objeto de estudio.

Los resultados presentados en esta Tesis Doctoral muestran diferentes respuestas según la arcilla seleccionada. De tal forma, CNa^+ mostró una respuesta positiva en la inducción de MN y otras anomalías celulares, como brotes nucleares, a concentraciones no citotóxicas (0-62,5µg/mL) tras 24h de exposición en células HepG2; sin embargo, no se observó una respuesta mutagénica mediante la exposición de 5 cepas de *Salmonella typhimurium* a concentraciones superiores de la misma (hasta 125µg/mL). Por otro lado, las arcillas modificadas disponibles comercialmente, C30B y C20A, mostraron un perfil genotóxico muy diferente. C20A indicó ausencia de genotoxicidad mediante el ensayo cometa en las células HepG2 y Caco-2, mientras que, C30B indujo rotura de las hebras de ADN en la línea hepática (0-88µg/mL) pero no en la línea intestinal (0-40µg/mL) con las condiciones ensayadas. Sin embargo, no se observó daño genotóxico mediante el ensayo MN a concentraciones subcitotóxicas (0-31,25µg/mL) en células HepG2 aunque sí daño mutagénico mediante el test Ames, siendo en este último más elevadas las concentraciones ensayadas (0-250µg/mL). En el caso de Clay1 (0-8µg/mL), solamente se obtuvo una respuesta positiva en el caso del test de Ames en presencia de fracción metabólica externa, mientras que no produjo genotoxicidad bajo las condiciones de los ensayos de MN y cometa. Clay2 causó daño en el ADN en células HepG2 y Caco-2 mediante el ensayo cometa (0-88 y 0-34µg/mL, respectivamente), así como cambios significativos en ensayo de MN y test de Ames con respecto al grupo control (0-15,6 y 0-125 µg/mL para ambos ensayos, respectivamente).

Li y col. (2010) coincidieron con la respuesta obtenida por Mt (0-1000µg/ placa) en el test de Ames, no mostrando diferencias significativas con el grupo control. Por el contrario, Taylor y col. (2014) informaron del incremento de colonias revertientes de *Salmonella typhimurium* expuestas a CNa^+ (0-0.01g), indicando potencial mutagénico de la misma. Los resultados obtenidos en nuestro estudio se contraponen también a los publicados por Sharma y col. (2010), que observaron efectos genotóxicos en Caco-2 expuestas a C30B (0-226µg/mL) mediante el ensayo cometa, siendo superiores las concentraciones ensayadas en este caso.

Otros autores, incluyendo la EFSA, evaluaron también el potencial genotóxico presente en bentonitas nativas y modificadas. Los datos disponibles informan de una ausencia de genotoxicidad de la bentonita nativa, confirmada mediante el ensayo de MN, test de Ames y enyaso cometa, frente al potencial genotóxico presentado por la bentonita modificada con H_2SO_4.

En cuanto a Clay3, Clay4A y Clay4B se obtuvieron resultados muy variados, desde la ausencia de genotoxidad (ensayo Cometa) y mutagenicidad por exposición de Clay3 y Clay4A en HepG2 y Caco-2, y, *Salmonella typhimurium*, respectivamente, hasta la repuesta positiva de Clay4B en ambos ensayos mencionados bajo las condiciones ensayadas. En la literatura científica no existen estudios de genotoxicidad realizados con arcillas modificadas con silanos.

Por otro lado, entre los biomarcadores más comúnmente ensayados en relación al proceso inflamatorio se encuentran la liberación de citoquinas, principalmente interleucina-6 (IL-6) y el factor de necrosis tumoral α (TNF-α). Hasta el momento, los estudios de inflamación levados a cabo con arcillas son muy escasos. Únicamente, Elmore y col. (2003) informaron de la producción de citotoxicidad en células macrófagas así como actividad hemolítica en células sanguíneas de diferentes especies generadas tras la exposición a diversas arcillas, entre ellas Mt. Además, se ha publicado que la Mt promueve infección mediante citotoxicidad directa en neutrófilos, dejándoles inservibles para la fagocitosis bacteriana (Dougherty y col., 1985). Estos resultados no coinciden con los obtenidos en nuestro estudio, donde HepG2 y Caco-2 expuestas a C30B y Clay2 no mostraron un incremento en la liberación de la IL-6.

Otros parámetros a tener en cuenta son los diferentes marcadores utilizados para determinar el mecanismo de muerte celular, destacando entre ellos la citometría de

flujo, que informa del porcentaje de células que sufren muerte celular programada (apoptosis o necrosis) y la actividad de las enzimas caspasas indicativa de apoptosis (Napierska y col., 2009; Lordan y col., 2011).

En nuestro estudio se obtuvo una respuesta de la actividad caspasa 3/7 de células HepG2 expuestas a C30B (0-500µg/mL). Estos resultados son similares a los obtenidos por Lordan y col. (2011) en células HepG2 expuestas a CNa^+ y C93A (0-1000µg/mL), informando de unos niveles no significativos con respecto el grupo control. Recientemente, Janer y col. (2014) han diferido de los resultados obtenidos hasta el momento, encontrando diferencias de la actividad caspasa 3/7 así como alteraciones mediante citometría de flujo de células HepG2 y SKMEL en presencia de Mt y arcillas modificadas con sales cuaternarias de amonio (0-500µg/mL). Estos resultados concuerdan con los publicados por Liu y col. (2010), los cuales describieron un incremento de la actividad caspasa e inducción de apoptosis en células NIH3T3 y HEK293 tras exposición a Mt y Mt modificada con oligo(estireno-co-acrilonitrilo) (1g/L). Además, Meibian y col. (2010) observó en células HMyR2 una inducción de apoptosis mediante citometría de flujo por exposición a partículas de bentonita, arcillas muy similares a la Mt.

Por otro lado, Caco-2 fueron expuestas a Clay4A (0-250µg/mL) y Clay4B (0-40µg/mL), observándose un mecanismo de apoptosis y necrosis tras la exposición a ambas, respectivamente, mediante citometría de flujo.

La expresión génica y abundancia proteica son dos marcadores imprescindibles para determinar que está ocurriendo realmente a nivel molecular tras la exposición a diversas sustancias. Los datos obtenidos por la exposición de concentraciones subcitotóxicas de CNa+ y Clay2 a HepG2 demuestran una importante desregulación génica de genes involucrados en la activación/ destoxicación así como en mecanismos de toxicidad, mientras que, no se obtuvo una significativa sobreexpresión o baja regulación de genes con respecto al control en el caso de C30B y Clay1. Actualmente, no existen en bibliografía trabajos acerca del potencial de modulación en líneas celulares eucariotas que presentan las arcillas destinadas al envasado sobre la regulación génica; sin embargo, Abatte y col. (2009) estudiaron la influencia de arcillas comerciales, entre ellas C30B, sobre la comunidad eurobacteriana presente en el suelo. Tras la exposición

de C30B se observaron únicamente alteraciones leves en *Pseudomonas putida*, pero no en el resto de bacterias ensayadas.

En relación a la toxicidad *in vitro* de los materiales nanocompuestos y extractos de migración obtenidos a partir de ellos, la información disponible es mucho más escasa. La citotoxicidad y mutagenicidad de los extractos de migración generados a partir de ácido poli(láctico) (PLA) y Clay1, y, PLA-Clay2, y, polipropileno (PP)- Clay3, han sido evaluadas en nuestras investigaciones, no observando efectos citotóxicos en HepG2 y Caco-2 mediante tres biomarcadores ensayados (RN, MTS y PT) en un rango de concentraciones de 0-100%. Del mismo modo, *Salmonella typhimurium* no mostró un incremento de colonias revertientes tras la exposición a ambos simulantes. Además, se ha observado un incremento de la vida útil de carnes de aproximadamente 24h, indicando la compatibilidad y mejora de los materiales resultantes. Otros autores han descrito también la inexistencia de efectos citotóxicos y cambio morfológicos, así como la buena biocompatibilidad de materiales nanocompuestos investigados en diferentes líneas celulares (NIH3T3, L929, IMR32, fibroblastos y osteoblastos) durante periodos de exposición generalmente amplios (1-7 días) (Zia y col., 2011; Liu y col., 2012; Fukushima y col., 2012, 2013; Kevadiya y col., 2013, 2014). La generación de estrés oxidativo también fue menor en aquellos grupos expuestos a materiales microcompuestos de polímero y arcillas modificadas (Kevadiya y col., 2013, 2014). Hasta el momento no existen en la literatura científica más estudios acerca del potencial genotóxico de extractos o materiales nanocompuestos; sin embargo, podemos encontrar algunos trabajosde genotoxicidad y mutagenicidad de modificadores potenciales, mostrando tanto efectos positivos como negativos (Dmochowska y col., 2011; Grabinska-Sota, 2011).

Por último, nos resultó de interés evaluar el contenido de la migración de los metales más característicos que se encuentran en la estructura de la Mt (Al, Ca, Fe, Mg y Si) en los simulantes alimentarios utilizados que constituyen los extractos de migración de PLA-Clay1, PLA-Clay2, siendo en este caso agua destilada, y, PP-Clay3 y PP-Clay4B, utilizándose para ello etanol al 10% e isooctano. No se encontraron diferencias significativas con respecto al grupo control en el caso de los extractos obtenidos a partir de PLA-Clay1 y PLA-Clay2; sin embargo, se obtuvieron diferencias significativas en los contenidos de Al, Ca y Si de los simulantes empleados en PP-Clay3 y PP-Clay4B. Podemos hallar en la literatura científica trabajos que evalúan la

migración de metales de materiales nancompuestos a diversos simulantes y/o alimentos. Avella y col. (2005) estudiaron la migración de metales a lechuga y espinacas en contacto con materiales nanocompuestos de almidón de patata y Mt. No se observaron diferencias significativas en los contenidos de Fe y Mg, mientras que sí se observó un mayor contenido de Si, siendo fácilmente atribuible al alto contenido de este metal que presenta a arcilla. Por otro lado, Busolo y Lagaron (2012) investigaron la migración de Al y Fe de materiales nanocompuestos de poliolefinas con caolinitas. Los simulantes seleccionados fueron agua e isooctano, no hallándose diferencias significativas en ninguna de las muestras ensayadas con respecto al control.

De forma resumida podemos concluir este apartado que los resultados obtenidos por los diferentes autores están principalmente influidos por los diferentes parámetros: a) modelo experimental seleccionado; b) concentraciones y tiempos de exposición ensayados; c) modificadores incorporados (si los hubiere) y d) sensibilidad de los biomarcadores ensayados.

2. ESTUDIOS *IN VIVO* SOBRE ARCILLAS Y MATERIALES NANOCOMPUESTOS

El número de estudios experimentales de toxicidad *in vivo* realizados recientemente con arcillas en la literatura científica es bajo en comparación con los estudios *in vitro*, obteniéndose una amplia variabilidad de resultados dependiendo de la selección de la arcilla, las rutas de exposición y concentraciones utilizadas.

Los estudios llevados a cabo con arcillas nativas, como es el caso de la Mt, mostraron de forma general una ausencia de efectos histopatológicos después de una exposición aguda (Baek y col., 2012; Lee y col., 2005; Mascolo y col., 2004) o una exposición subcrónica o crónica en roedores (Afrie-Gyawu y col., 2005; EFSA, 2013), siendo éste último caso el que corresponde a los estudios realizados en la presente Tesis Doctoral.

De tal forma, Baek y col. (2012) estimaron una dosis letal media (DL50) de la Mt superior a 1000mg/Kg en ratones tras 14 días de exposición. La ausencia de toxicidad o la obtención de efectos mínimos de toxicidad coincide con lo expuesto por Wiles y col. (2004), los cuales no observaron diferencias significativas entre embriones de ratas

embarazadas Sprague-Dawley expuestas a Mt (2%w/w) y el grupo control. Por el contrario, Patterson y Stasak (1977) publicaron los efectos negativos encontrados en ratas madre Sprague-Dawley expuestas a 20% de caolín durante el periodo de gestación, observando alteraciones en el desarrollo, anemia materna y una considerable reducción en el peso de nacimiento de las crías. Sin embargo, el consumo de arcillas por animales salvajes en la naturaleza es común, estando dicha práctica bien documentada (Slamova y col., 2011). Se han propuesto varias hipótesis para explicar el comportamiento geofágico (comer "arcillas"): (i) la desintoxicación de compuestos nocivos o desagradables presentes en la dieta, (2) alivio de trastornos gastrointestinales, tales como diarrea, (3) la suplementación del cuerpo con minerales y (4) alivio de la hiperacidez en el tracto digestivo (Wilson, 2003). De hecho, existen informes disponibles acerca de la capacidad de la arcilla para reducir micotoxicosis, metales, aliviar trastornos gastrointestinales etc., como se ha explicado anteriormente la Introducción.

En nuestro caso, una vez seleccionada la arcilla de mejor perfil tecnológico y toxicológico, Clay1, realizamos un estudio de toxicidad subcrónica de 90 días en ratas Wistar expuestas a Clay1 en la comida (40 mg/kg/día) y a su extracto de migración como agua de bebida. La inexistencia de cambios histopatólogicos con respecto al grupo control tras la exposición a arcillas descrita con anterioridad coinciden con los datos presentados en nuestras investigaciones, no observándose ningún daño en el grupo de órganos estudiados. Además, en nuestro estudio, la ausencia de efectos tóxicos fue recogida de forma general con todos los parámetros evaluados, siendo únicamente significativa la actividad de la enzima catalasa (CAT) en riñón, así como su expresión génica y abundancia proteica tras exposición a Clay1. Ningún cambio fue observado en el caso del extracto de migración. En cuanto al estudio de biomarcadores de estrés oxidativo podemos encontrar los datos publicados por Kibanova y col. (2009), los cuales informan de un incremento de peroxidación lipídica (LPO) en sobrenadante de cerebros de roedores expuestos a hectorita (1000ppm).

En relación a la genotoxicidad, la EFSA (2013) ha publicado en su informe sobre la bentonita la no detección de aberraciones cromosómicas tras su exposición en ratas durante 15 días. Del mismo modo, Sharma y col. (2014) tampoco observaron rotura en la hebras de ADN en colon, hígado y riñón de ratas Wistar expuestas mediante sonda a C30B (250-1000mg/kg/ peso corporal). Igualmente, también informaron de la ausencia

de respuestas inflamatorias tras exposición a la arcilla modificada. Estos últimos resultados coinciden con los revelados por Hsu y col. (2012), los cuales expusieron mediante inyección subcutánea a ratas Sprague-Dawley a Mt sin la obtención de respuestas inflamatorias en los tejidos.

La Agencia Internacional de Investigación contra el Cáncer (IARC) ha evaluado la sepiolita con el fin de clasificarla según su potencial carcinogénico, quedando finalmente situada en el Grupo 3 (no puede ser clasificado respecto a su carcinogenicidad para el ser humano), basado esto en la información tan limitada que hay acerca de la carcinogenicidad producida tras largos periodos de exposición en animales (IARC, 1997).

Algunos autores justifican la ausencia de efectos tóxicos a la escasez de estudios sobre la exposición sistémica (Sharma y col., 2014). Sin embargo, Baek y col. (2012) no observaron toxicidad alguna incluso a concentraciones mucho más elevadas de las utilizadas normalmente, 1000mg/kg de peso corporal, informando que la arcilla puede ser absorbida en un periodo de 2h sin acumulación en ningún órgano específico.

Para investigar la acumulación de minerales de arcilla realizamos la cuantificación del contenido de los metales estructurales característicos (Al, Ca, Fe, Mg y Si) en bazo de ratas expuestas a Clay1 y su extracto de migración, no obteniendo diferencias significativas con respecto el grupo control. En este sentido, se han encontrado resultados contradictorios en la literatura científica. Mascolo y col. (1999, 2004) observaron un aumento de la acumulación progresiva de los metales en la orina y diferentes tejidos de ratas en el siguiente orden: riñón> hígado> corazón> cerebro. Además, Reichardt y col. (2007) informaron que la caolinita se disocia en el lumen y que el Al pasa directamente a través de la barrera intestinal. En contraste, Sharma y col. (2014) no encontraron un aumento en el contenido de Al en el hígado y los riñones de las ratas expuestas por sonda a 1000mg/kg de peso corporal a C30B. La EFSA (2013) también consideró que la bentonita y sepiolita, de manera similar a otras arcillas, no son absorbidas en una medida cuantificable.

Respecto a la evaluación toxicológica de materiales nanocompuestos, a parte de los resultados obtenidos en nuestro ensayo, no obteniendo diferencia alguna con respecto al control en los parámetros ensayados de ratas Wistar expuestas al extracto de migración de Clay1, solamente encontramos en bibliografía un trabajo disponible. Hsu y col.

(2012) publicaron el aumento de biocompatibilidad de un material nanocompuesto de de Mt/ quitosano con respecto al material de partida en un ensayo llevado a cabo con ratas Sprague-Dawley.

3. ESTUDIOS DE GENOTOXICIDAD SOBRE ACEITES ESENCIALES Y COMPONENTES MAYORITARIOS

Por todo lo expuesto a lo largo de este Tesis Doctoral, la evaluación toxicológica de materiales que van a entrar en contacto con alimentos es necesaria. El uso en el envasado de aceites esenciales y componentes mayoritarios es una tendencia en auge. En este caso, el aceite esencial de orégano y, más específicamente, sus componentes mayoritarios, timol y carvacrol, son un ejemplo de ello. Estudios sobre el riesgo de estos compuestos mayoritarios se han realizado con anterioridad, no siendo concluyentes los resultados obtenidos, sobre todo en el caso del carvacrol. En este sentido, son necesarias más evaluaciones para determinar los riesgos potenciales asociados a estos compuestos y su uso en la industria alimentaria. Por ello, nos propusimos a investigar el potencial genotóxico de timol y carvacrol mediante el ensayo de MN y el ensayo de linfoma de ratón (MLA), realizándose éste último por primera vez dada la inexistencia de resultados publicados al respecto en la literatura científica. Los datos obtenidos demostraron que el timol no dio lugar a una respuesta genotóxica mediante los ensayos de MN y el MLA con las concentraciones enyasadas, destinadas las mismas a ser incorporadas en el envasado de alimentos. Este hecho se ve reforzado por los resultados obtenidos por Llana-Ruíz-Cabello y col. (2014), los cuales mostraron ausencia de daño en el ADN mediante el ensayo cometa en células Caco-2 y una respuesta negativa en el test de Ames en cepas de *Salmonella typhimurium* bajo las condiciones ensayadas. Además, otros autores también coinciden con estos resultados, describiendo una respuesta no significativa por exposición de timol mediante el ensayo cometa en otras líneas celulares tales como Caco-2 (intestinales) o V79 (fibroblastos) (Horvathová y col., 2006; Undeger y col., 2009). Por el contario, nuestros resultados se contraponen a los publicados por Buyukleyla y Rencuzogullari (2009), quienes informaron del aumento significativo en la frecuencia de intercambios de cromátidas hermanas (SCE) e inducción de aberraciones cromosómicas y MN en linfocitos humanos a todas las concentraciones ensayadas (25-100 mg/ml). La ausencia de genotoxicidad observada

para timol en el presente trabajo se podría atribuir a sus propiedades antioxidantes ampliamente probadas (Aydin y col., 2005a; Slamenova y col., 2007; Buyukleyla y Rencuzogullari, 2009).

En relación al carvacrol, éste exhibió un ligero potencial genotóxico en el ensayo de MN, en la concentración más alta ensayada (700 mM) en células L5178Y sin presencia de fracción metabólica externa, y, no se encontró ninguna respuesta significativa en el ensayo MLA después de 4 y 24h de exposición. Este resultado indica un riesgo genotóxico bajo, pudiendo ser considerado incluso como marginal y sin relevancia biológica. Podemos comparar nuestros resultados de MN con los previamente obtenidos por la prueba de Ames (con y sin S9) (Llana-Ruíz-Cabello y col., 2014). En este ensayo, carvacrol muestra una mayor actividad genotóxica en presencia de la fracción metabólica y a bajas concentraciones (29-460 M). Para resolver esta discrepancia entre ensayos, Kirkland y col. (2014) propone que las diferencias entre ambos ensayos, Ames y MN, podrían explicarse debido a las diferencias en el metabolismo de células bacterianas y de mamíferos. Estos autores propusieron que 1) las concentraciones de S9 que se usan en casa ensayo son diferentes; 2) la duración de la exposición es más larga en el test de Ames (72h) en comparación con las pruebas de células de mamíferos (3-6 h en el ensayo de MN); 3) el metabolismo intrínseco de las células bacterianas y de mamíferos puede producir diferentes perfiles metabólicos; y 4) la mezcla S9 favorece el metabolismo oxidativo, el cual las células de mamíferos son capaces de contrarrestar a través del metabolismo de fase II para promover la desintoxicación, mientras que las bacterias pueden ser menos capaces de defenderse contra este tipo de daño.

Por otra parte, en bibliografía podemos encontrar una importante disparidad de los resultados obtenidos por carvacrol en diferentes modelos experimentales. Stammati y col. (1999) indicaron una ausencia de mutagenicidad de este monoterpeno mediante el Test de Ames, mientras que otros estudios informaron del potencial antimutagénico (Ipek y col., 2005). Por otro lado, Aydin y col. (2005a, b) informaron de una respuesta genotóxica positiva en linfocitos humanos mediante el ensayo cometa, mientras que otros autores han descrito la falta de genotoxicidad a través del mismo ensayo en líneas mamíferas tales como Caco-2, HepG2, V79 y N2a (Undeger y col., 2009; Aydin y col., 2014).

Los resultados obtenidos en la presente Tesis Doctoral serán de gran interés para el futuro empleo de las sustancias estudiadas, arcillas y componentes mayoritarios de aceites esenciales, en el envasado de alimentos salvaguardando la salud de los consumidores.

VI. CONCLUSIONES/ CONCLUSIONS

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones:

PRIMERA. Los perfiles de toxicidad de los minerales de arcilla y nanocompuestos derivados se definen de acuerdo con diferentes parámetros, incluyendo (i) las condiciones de exposición, tales como las concentraciones o tiempos ensayados, (ii) los modelos experimentales seleccionados, (iii) los modificadores o agentes tensioactivos incorporados en sus estructuras y sus concentraciones, (iv) la sensibilidad de los ensayos realizados, etc. Por todo ellos se hace necesaria una evaluación toxicológica caso por caso.

SEGUNDA. La evaluación *in vitro* en la línea celular de hepatoma humano (HepG2) expuesta a la arcilla no modificada, CNa^+ y diversas arcillas organomodificadas con sales de amonio cuaternario (C30B, C20A, Clay1 y Clay2), dio lugar a diferentes respuestas según el material objeto de estudio ensayado tras 24 y 48h de exposición. De forma general, no se observó ningún efecto citotóxico tras la exposición a CNa^+, aunque sí pudo observarse una respuesta positiva en el ensayo de MN. En el caso de C30B se obtuvo daño citotóxico acompañado además de alteración de la morfología celular, daño genotóxico mediante el ensayo cometa y disminución de contenido en GSH. La línea HepG2 expuesta a C20A y Clay1 presentó una ausencia de toxicidad de forma general; sin embargo, Clay2 produjo una disminución de la viabilidad celular, inducción de rotura de la hebra de ADN y genotoxicidad en el ensayo de MN, y, descenso en el contenido de GSH.

TERCERA. Se ha demostrado por primera vez que concentraciones subcitotóxicas de CNa^+, C30B, Clay1 y Clay2 modulan la expresión génica en células HepG2. Únicamente se observaron importantes desregulaciones en la expresión de genes implicados en la activación/destoxicación y diferentes mecanismos de acción, tales como respuesta al daño en el ADN y muerte celular, en el caso de CNa^+ y Clay2.

CUARTA. Se ha comprobado también el efecto tóxico de las arcillas CNa^+, C30B, C20A, Clay1 y Clay2 en la línea de adenocarcinoma humano Caco-2, no obteniendo de forma general efectos citotóxicos y genotóxicos tras la exposición a CNa^+ y Clay1. No obstante, tanto C30B como Clay2 mostraron un descenso en la viabilidad celular y daño morfológico (C30B), y en el caso de Clay2, un efecto genotóxico mediante el ensayo cometa.

QUINTA. De forma similar, se encontró una baja respuesta tóxica en la línea celular endotelial de vena umbilical (HUVEC) tras la exposición por primera vez a CNa^+ y Clay1,

aunque se observaron efectos citotóxicos tras la exposición a C30B y Clay2 en todos los biomarcadores ensayados tras 24 y 48h de exposición.

SEXTA. La evaluación del potencial mutagénico mediante el Test de Ames de CNa^+, C30B, Clay1 y Clay2 ha demostrado una ausencia de mutagenicidad en el caso de la arcilla no modificada y de Clay2. No obstante, Clay1 manifiesta un efecto tóxico notable, presentando respuesta mutagénica en presencia de activación metabólica en una de las cepas ensayadas, TA102. Del mismo modo, C30B presentó un aumento en las colonias revertientes de la cepa TA98 de *Salmonella typhimurium*, también en presencia de la fracción metabólica S9.

SÉPTIMA. Se ha demostrado la ausencia de efectos citotóxicos en las líneas HepG2 y Caco-2 tras la exposición a extractos de migración obtenidos a partir de PLA con Clay1 y PLA con Clay2. Además, la ausencia de potencial mutagénico en *S. typhimurium* también fue observada tras la exposición a ambos extractos. Por otro lado, no se constató migración de los metales mayoritarios presentes en la estructura de las arcillas (Al, Ca, Fe, Mg, Si) en ambos extractos.

OCTAVA. Tras la realización de un ensayo de toxicidad subcrónica durante 90 días en ratas Wistar expuestas a Clay1 (40 mg/kg/día), se ha comprobado la ausencia de daño hitopatológico en diversos órganos (hígado, riñón, intestino, corazón, cerebro, pulmones, testículos y bazo), así como alteraciones en la bioquímica clínica, liberación de interleucina-6 y ratio GSH/GSSG. Además, fueron estudiados en hígado y riñón los niveles de LPO (MDA), la actividad de las enzimas antioxidantes SOD, CAT, GPx y GST, y, la expresión génica y abundancia proteica de SOD y CAT. Únicamente se observaron cambios significativos en la actividad antioxidante, expresión génica y abundancia proteica de CAT en riñón.

NOVENA. Ratas Wistar expuestas durante 90 días al extracto de PLA-Clay1 como agua de bebida no mostraron ningún tipo de alteración en todos los parámetros considerados en el estudio, incluyendo: análisis histopatológico, liberación de IL-6, biomarcadores de estrés oxidativo (GSH/GSSG, LPO, CAT, SOD, GPx, GST), análisis de expresión génica y abundancia proteica de SOD y CAT. Además, se evaluó el contenido de Al, Ca, Fe, Mg y Si en bazo de ratas expuestas a Clay1 y su extracto de migración, no observándose cambios significativos.

DÉCIMA. Los resultados preliminares demuestran que el desarrollo de tres arcillas modificadas con silanos, Clay3, Clay4A y Clay4B, ha resultado ser exitoso dada la presencia

de los modificadores en la estructura de la arcilla, constatado mediante espectroscopía infrarroja (FTIR), difracción de rayos X y termogravimetría (TGA). Además, se analizó la migración de Al, Ca, Fe, Mg y Si en simulantes en contacto con PP-Clay3 y PP-Clay4B (etanol al 10% e isooctano). Se obtuvieron diferencias significativas con respecto al control y Clay3 en Ca, Mg y Si en el caso de etanol al 10%, y, de Si en el caso de isooctano. Los extractos de migración de Clay4A presentaron diferencias en Al y Ca en etanol al 10% y, Ca y Mg en isooctano. Los envases de PP-Clay3 mejoraron la vida útil de los alimentos (carne) en 24h.

UNDÉCIMA. Los resultados preliminares sugieren que Clay3 no induce descenso en la viabilidad de las células HepG2 y Caco-2. Sin embargo, tras la exposición de Clay4A se observaron efectos citotóxicos en las líneas hepática e intestinal, además de un incremento en la producción de ERO en ambas líneas celulares. Por otro lado, Clay4B es la arcilla que peor perfil toxicológico presenta de las tres sintetizadas. Dicha arcilla ha dado lugar a un descenso en la viabilidad celular, potencial genotóxico mediante el ensayo cometa y disminución del contenido de GSH en las líneas celulares ya mencionadas. Sólo se observó un incremento de ERO en el caso de HepG2 tras exposición a Clay4B. En cuanto al potencial mutagénico, fue la única que presentó una respuesta positiva, obteniéndose índices mutagénicos superiores a 2 en el caso de TA102 en ausencia y presencia de S9.

DUODÉCIMA. Se ha demostrado tras la revisión bibliográfica la disparidad de los resultados publicados hasta el momento sobre el efecto antioxidante y antimicrobiano de los aceites esenciales y componentes mayoritarios incorporados a envases, pudiendo ser debido a: (i) factores inherentes de los aceites esenciales, (ii) películas plásticas, (iii) condiciones ensayadas, (iv) tiempos de exposición, etc. En este sentido, es necesario establecer un rango de concentraciones de uso apropiado con el fin de asegurar la salud de los consumidores.

DÉCIMOTERCERA. Se ha investigado el potencial genotóxico de timol y carvacrol, componentes mayoritarios del aceite esencial de orégano, mediante el ensayo de MN y el ensayo de Linfoma de Ratón. Se ha observado a las condiciones ensayadas la ausencia de potencial genotóxico de timol, sin embargo, carvacrol presentó diferencias significativas con respecto al control en la concentración más alta ensayada únicamente en el ensayo de MN. Teniendo en cuenta la información previa obtenida acerca de carvacrol en otros ensayos de genotoxicidad, incluidos los observados en el ensayo de linfoma de ratón, podrían considerarse los resultados genotóxicos obtenidos biológicamente no relevantes.

The main conclusions that can be drawn on the basis of the obtained results in the present Thesis are:

FIRST. The toxicity profiles of clay minerals and derived nanocomposites are defined according to different parameters, including (i) exposure conditions such as time or concentrationstested , (ii) the selected experimental models, (iii) modifiers or surfactants incorporated in their structures and their concentrations,(iv) the sensitivity of the tests performed, etc. For all these aspects a case by case toxicological assessment is necessary.

SECOND. The *in vitro* evaluation in human hepatoma cell line (HepG2) exposed to the unmodified clay, CNa^+ and several quaternary ammonium salts-modified clays (C30B, C20A, Clay1 and Clay2), gave different results depending on the material under study tested after 24 and 48h exposure. In general, no cytotoxic effects after exposure to CNa^+ were observed, although a positive response in the MN test was observed. Regarding to C30B, cytotoxic damage, alteration in cell morphology, genotoxic damage studied by the comet assay and decrease in GSH content were observed. HepG2 cell line exposed to C20A and Clay1 generally presented an absence of toxicity; however, Clay2 produced a cell viability decrease, induction of DNA strand breaks and genotoxicity observed by the comet and MN assays, respectively, and decrease in GSH content.

THIRD. For first time, the modulation of gene expression after subcytotoxic concentrations of CNa^+, C30B, Clay1 and Clay2 have been demonstrated. Important deregulations in the expression of genes involved in the activation/detoxification and different mechanisms of action, such as response to DNA damage and cell death, were only observed in the case of CNa^+ and Clay2.

FORTH. The toxic effects of the adenocarcinoma Caco-2 cell line exposed to the CNa^+, C30B, C20A, Clay1 and Clay2 were showed. In general, cytotoxic and genotoxic effects after exposure of CNa^+, C20A and Clay1 were not obtained. However, C30B and Clay2 induced a decrease in cell viability and morphological damage (C30B), and genotoxicity by comet assay in the case of Clay2.

FIFTH. Similarly, a low toxic response in human umbilical vein endothelial cells (HUVEC) exposed to CNa^+ and Clay1 was found, although cytotoxic effects after C30B and Clay2 exposure in all the biomarkers tested after 24 and 48h were observed.

SIXTH. The mutagenic potential of CNa^+, C30B, Clay1 and Clay2 has been evaluated by the Ames Test, being demonstrated a lack of mutagenicity with the non modified clay an Clay2; however, Clay1 showed a remarkable toxic effect, presenting mutagenic response in the strains tested (TA102) in the presence of metabolic activation. Similarly, C30B produced an increase in revertant colonies of *Salmonella typhimurium* strain TA98, also in the presence of S9 metabolic fraction.

SEVENTH. The absence of cytotoxic effects in HepG2 and Caco-2 cell lines exposed to the migration extracts obtained from PLA-Clay1 and PLA-Clay2 has been showed. Moreover, the lack of mutagenic potential in *S. typhimurium* after exposure to both extracts was also observed. Furthermore, no migration of the major metal present in the structure of the clay (Al, Ca, Fe, Mg, Si) was found in both extracts**.**

EIGHTH. Once the repeated dose 90-day oral toxicity study in Wistar rats exposed to Clay1 (40mg/kg/day) has been conducted, absence of histopathological damage in several organs (liver, kidney, intestine, heart, brain, lungs, testicles and spleen) was observed, as well as, no changes in clinical biochemistry, interleukin-6 release and alterations in GSH/GSSG ratio. Additionally, levels of LPO (MDA), activities of antioxidant enzymes SOD, CAT, GPx and GST, gene expression and protein abundance of SOD and CAT in liver and kidney were determined. Only significant changes in CAT antioxidant activity in kidney, as well as, gene expression and protein abundance, were demonstrated.

NINTH. Wistar rats exposed to PLA-Clay1 migration extract by drinking water during 90 days did not show any alterations in all the parameters considered, including: histopathological analysis, IL-6 leackage, oxidative stress biomarkers (GSH/GSSG, LPO, CAT, SOD, GPx, GST), gene expression analysis and protein abundance of SOD and CAT. Moreover, the migration content of Al, Ca, Fe, Mg and Si in the spleen of rats exposed to Clay1 and its migration extract was determined and no significance differences were observed.

TENTH. Preliminary results showed a successful development of three silane-modified clays due to the presence of modifier in clays structure, evaluated by: infrared spectroscopy (FTIR), X-ray diffraction and thermogravimetry (TGA). Furthermore, migration of Al, Ca, Fe, Mg and Si was analyzed in PP-Clay3 and PP-Clay4 simulants (ethanol 10% and isooctane). Significant differences between from control Clay3 were obtained in Ca, Mg and Si in the

case of ethanol 10%, and in Si with the isooctane simulant. Clay4A migration extracts presented significant changes in Al and Ca with ethanol 10% and, in Ca and Mg with isooctane. PP-Clay3 packages showed an improved of food shelf-life (meat) of 24h.

ELEVENTH. Preliminary results suggest no induction in cell viability decrease produced by Clay3 in HepG2 and Caco-2 cells. However, cytotoxic effects and an increase in ROS generation in both cell lines after Clay4A exposure were obtained. Clay4B is the clay with the worst toxicological profile, giving a cell viability decrease, genotoxicity by the comet assay and GSH content depletion in the above mentioned cell lines. An increase in ROS was only observed in HepG2 exposed to Clay4B. Moreover, a positive mutagenic potential was determined with Clay4B, obtaining mutagenic indexes higher than 2 in TA102 strain in absence and presence of S9.

TWELFTH. A disparity of the results published about the antioxidant and antimicrobial properties presented in active packaging obtained from essential oils and main compounds, as well as, their cytotoxic effects in human cell lines were found. They could be due to: (i) different factors inherent to the essential oils, (ii) the film, (iii) the conditions assayed, (iv) exposure times, etc. In this sense, it is important to establish an appropriate concentration range for their uses, based on toxicity studies, to ensure the safety of the consumers.

THIRTEENTH. Genotoxic potential has been investigated of thymol and carvacrol, two main compounds of oregano essential oil, by the MN and mouse lymphoma assays. An absence of genotoxic potential of thymol under the conditions tested was demonstrated; however, significant differences from the control at the highest concentration assayed with carvacrol in MN test were presented. Taking into account the previous information about carvacrol genotoxicity observed through other tests, including mouse lymphoma assay, the results obtained could be considered not relevant biologically.

VII. OTROS MÉRITOS/ OTHER MERITS

2013

Temas de interés en SEGURIDAD ALIMENTARIA

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RIESGOS DERIVADOS DEL EMPLEO DE NANOARCILLAS EN EL ENVASADO DE ALIMENTOS

Sara Maisanaba Hernández

Área de Toxicología, Facultad de Farmacia, Universidad de Sevilla. C/ Profesor García González nº 2, C.P. 41012, Sevilla, España.

Resumen

La industria del envasado alimentario, para lograr ser cada día más competitiva e ir consiguiendo avances que les ofrezcan beneficios, está apostando por nuevos materiales que incorporados a los polímeros permitan incrementar la vida útil de los alimentos. Entre estos materiales se encuentran las arcillas organomodificadas con sales de amonio cuaternario, las cuales una vez imbuidas en el film de envasado adquieren una conformación en plaquetas de grosor nanométrico dando lugar a un material nanocompuesto. El objetivo de este trabajo es realizar una revisión de la evaluación toxicológica de estas nanoarcillas, las ya disponibles comercialmente y las que están desarrollándose en la actualidad, relacionándolas con el posible efecto que podrían tener en un futuro si el consumidor estuviese en contacto con ellas. Esta revisión se estructura en dos grandes bloques, por un lado los estudios *in vitro* llevados a cabo, subdivididos en ensayos de citotoxicidad, genotoxicidad y estrés oxidativo, así como los resultados obtenidos hasta el momento. Y, por otro lado, los estudios *in vivo*, claramente diferenciados en estudios a corto y largo plazo, obteniéndose a partir de ellos conclusiones muy diversas.

Abstract

The food packaging industry, in order to become more competitive and get new advances and benefits, is focused in the development of novel materials, which incorporated into the polymers allow to increase the shelf-life of food products. Among these materials the organomodified clays with quaternary ammonium salts show the major potential. When these clays are imbued in the packaging film, they are dispersed as platelets of nanometric size, giving a resultant material known as nanocomposite. The aim of this paper is to review the toxicological evaluation of these nanoclays, those

commercially available and those that are under development, identifying the potential effects they can induce if consumers in the future get in contact with them. This review is divided into two blocks, first, in vitro studies, subdivided into cytotoxicity, genotoxicity and oxidative stress assays, and the results obtained so far. On the other hand, in vivo studies, clearly differentiated in short and long term assays, obtaining from them very different conclusions.

Palabras clave: nanoarcillas, envases, toxicidad, *in vitro*, *in vivo*

Índice

1. Introducción

Los plásticos han reemplazado en el envasado de alimentos a los materiales usados más convencionalmente (el vidrio, la cerámica, el metal, el papel y el cartón) debido a su funcionalidad, peso ligero, bajo precio y la facilidad a la hora de ser procesados (Majeed y col., 2013). Sin embargo, no todo son ventajas, estos polímeros presentan una alta permeabilidad a gases y vapores, limitándose su uso para determinados alimentos (Arora y col., 2010). Debido a la debilidad que presentan, en los últimos años se han desarrollado unos nuevos polímeros conocidos como polímeros nancocompuestos (PNCs). Estos plásticos han sido diseñados con el fin de mejorar las deficiencias del material madre, añadiendo en su desarrollo materiales que hagan posible, entre otras cosas, un material menos permeable. Los PNCs más prometedores son aquellos compuestos por polímeros orgánicos y arcillas minerales inorgánicas. Entre las arcillas más utilizadas como nanorelleno se encuentra la montmorillonita (MMT) (Duncan y col., 2011), un hidroxisilicato de magnesio y aluminio. Esta arcilla presenta una estructura laminar de un grosor nanométrico (Chen y col., 2003) una vez incorporada al polímero, de ahí que el material resultante tome el nombre de material nanocompuesto. La MMT, comercialmente conocida como Cloisite®Na^+ (CNa^+), está compuesta por dos láminas tetraédricas formadas por silicio (Si) y oxígeno (O) acoplada a una lámina octaédrica formada por átomos de magnesio (Mg) y aluminio (Al) unidos a O y grupos hidroxilo (OH) (Jordá-Beneyto y col., 2008) (Fig. 1). Además, entre otras características, la MMT presenta una elevada área superficial y un amplio ratio tamaño-superficie, haciendo que sea un efectivo relleno de refuerzo (De Azeredo y col., 2009). También se caracteriza por una alta resistencia y rigidez, considerándose los materiales obtenidos como rígidos e impermeables (Majeed y col., 2013).

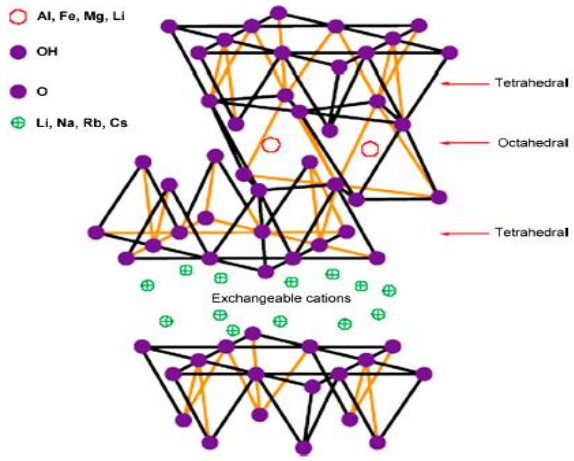

Figura 1. Estructura de la MMT (T, lámina tetraédrica; O, lámina octaédrica; C, cationes intercalados; d, distancia interlaminar) (Lagaly y col., 2005).

Un inconveniente importante relacionado con el desarrollo de materiales nanocompuestos mediante el uso de filosilicatos es la intercalación de las cadenas de la matriz polimérica entre las láminas de arcilla. La exfoliación de estas láminas de arcillas tiene un papel crucial, así como una uniforme dispersión, siendo esencial para un mejor refuerzo de los materiales nanocompuestos. Varios grupos de investigación han estudiado el efecto de la organofilización de los silicatos laminares, concluyendo que la energía superficial de las arcillas no tratadas, como es el caso de la MMT, es muy elevada, por lo que las fuerzas que mantienen unidas las láminas que la componen son muy fuertes (Kádar y col., 2006). Hay determinados agentes, como los tensioactivos, que incorporados a la arcilla dan lugar a una reducción de la energía superficial, siendo directamente proporcional al aumento en la separación de las láminas. Teniendo en cuenta la hidrofilia que presentan estas arcillas, frente a la hidrofobia de la mayoría de los polímeros, para que se dé una buena exfoliación debe llevarse a cabo una modificación química superficial, consiguiendo de esta forma un mejor resultado. Éste mayor rendimiento puede ser conseguido fácilmente por el intercambio de cationes de la superficie hidrófila de la arcilla (Na^+, K^+) con cationes orgánicos, como sales de amonio cuaternario. Ésta última modificación es la más común llevada a cabo con la MMT, tratada con alcaliamonios (Pavlidou y Papaspyrides., 2008). La figura 2 muestra la estructura química de algunas sales de amonio cuaternario utilizadas para la modificación de la MMT. La incorporación a la arcilla de estos modificadores da lugar a un descenso de la energía superficial entre las láminas del hidrosilicato, así como un refuerzo de la afinidad entre el polímero y la arcilla (Ráquez y col., 2013).

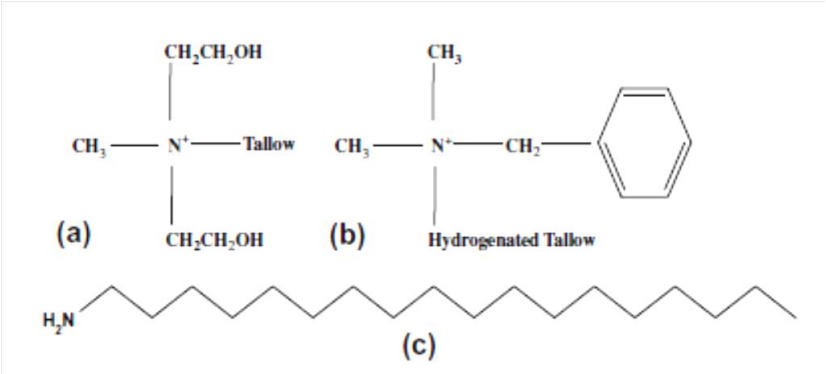

Figura2. Estructura química de organomodificadores: (a) metil, 2bis-hidroxietil, amonio cuaternario (b) dimetil, benzyl, hidrogenado amonio cuaternario (c) octadecilamina.

Una vez incorporadas las arcillas modificadas a los polímeros, deben presentar una buena exfoliación de las láminas, una distribución homogénea y estar orientadas en la dirección correcta constituyendo una fase continua. Finalmente se obtendrán unos polímeros nanocompuestos destinados a reducir la permeabilidad de los gases, como oxígeno y dióxido de carbono, vapores orgánicos y humedad, siendo la principal debilidad que presentan los polímeros convencionales (Silvestre y col., 2011). Muchos estudios han demostrado que los polímeros nanocompuestos de arcilla pueden retardar la migración de aditivos potencialmente dañinos del envase al alimento. En general, las plaquetas de arcillas son impermeables y la incorporación en la matriz polimérica da lugar a un paso tortuoso para una difusión lenta a través del material nanocompuesto (Fig. 3).

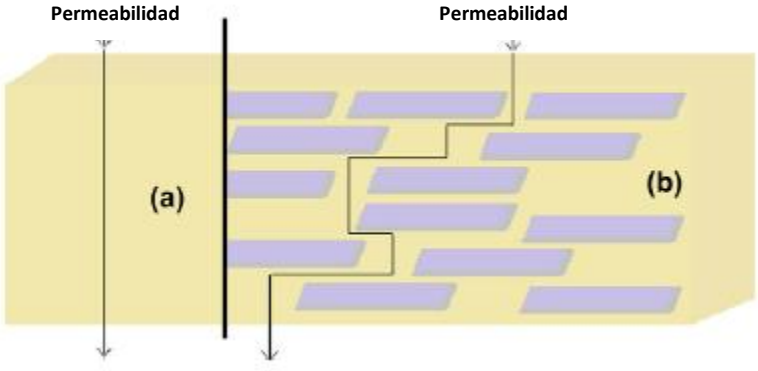

Figura 3. Difusión de una molécula permeable a través de (a) Film polimérico (b) Film nanocompuesto con una disposición exfoliada de las plaquetas impermeables de arcilla (Duncan, 2011).

En definitiva, la incorporación de arcillas a polímeros y la obtención de materiales nancompuestos van a suponer dentro de la industria alimentaria un campo en continuo

desarrollo, ya que supondría, a nivel industrial, una mejora destacada que conlleva ahorro en costes y pérdidas de productos.

En cuanto al aspecto tecnológico, es amplia la información disponible sobre estos nuevos materiales, por qué características destacan y cuál es el fin de los mismos. Sin embargo, en cuanto al riesgo del empleo de las arcillas como materiales de refuerzo en plásticos destinados a la industria alimentaria, la información es más escasa.

Debido al amplio espectro de aplicaciones que tienen estas arcillas organomodificadas, el consumidor cada vez va a estar más expuesto a ellas. Hasta el momento, la vía inhalatoria era la principal vía de entrada para que se produjesen intoxicaciones por arcillas minerales (Carretero y col., 2006). Sin embargo, al estar presente estas arcillas en materiales de envasado, la vía de entrada más común en la actualidad es la oral, pudiendo presentarse migración de las mismas desde el material de envasado hasta el alimento En la bibliografía existen estudios llevados a cabo tanto *in vitro* como *in vivo*, siendo los primeros más numerosos, centrados en arcillas provenientes de la MMT, ya sea la propia arcilla inicial o bien arcillas modificadas con las sales de amonio cuaternario anteriormente citadas. En dichos estudios se evalúan determinados marcadores que reflejan el efecto que pueden tener sobre el organismo, así como los posibles mecanismos de acción para dar lugar al efecto tóxico.

A continuación, se presenta una exhaustiva revisión de los resultados en materia de evaluación toxicológica publicados hasta el momento por distintos grupos de investigación, distinguiendo entre ensayos *in vitro* e *in vivo*.

2. Evaluación toxicológica *in vitro*

Los métodos *in vitro* proporcionan un medio efectivo y rápido para la selección (*screnning*) y clasificación de compuestos, además de ser ética y moralmente más correctos que los ensayos *in vivo*. Entre otras ventajas destacan que el uso de animales es considerablemente menor, e incluso nulo en muchas ocasiones, se utiliza material muy homogéneo obtenido con técnicas estandarizadas, posibilitan el uso de material de origen humano, lo que se puede simplificar por extrapolación, son más fácilmente objetivables y cuantificables que los ensayos *in vivo* y los resultados presentan mayor

reproducibilidad. A todo ello se le une un menor coste, mayor rapidez e instalaciones menos complejas.

Son varios los indicadores de toxicidad que se pueden determinar con los métodos *in vitro*, entre los que destacan los valorados en la bibliografía y que a continuación se exponen.

2.1. Ensayos de Citotoxicidad

Seleccionadas las líneas celulares de trabajo, los ensayos de citotoxicidad son los llevados a cabo en primera instancia para valorar los efectos adversos o interferencias con estructuras y/o propiedades esenciales para la supervivencia, proliferación y/o función celular una vez que se les ha expuesto el compuesto objeto de estudio.

Diversos grupos han evaluado mediante diferentes biomarcadores de citotoxicidad la alteración funcional producida por exposición de diferentes líneas celulares a dichas arcillas organomodificadas. El grupo de investigación de Li y col., 2010, estudió la citotoxicidad en una línea celular ovárica de hámster (CHO) expuesta a nanoláminas de silicato (NSP) de MMT. Los biomarcadores evaluados fueron el ensayo de viabilidad MTT (de acuerdo con las instrucciones del fabricante), que evalúa la capacidad mitocondrial para reducir el Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT), comprobando la funcionalidad mitocondrial tras la exposición al compuesto. Además, se estudió la liberación de la enzima Lactato Deshidrogenasa (LDH) para evaluar el daño en la membrana celular. Las células fueron expuestas a diferentes concentraciones de NSP (62,5; 125; 250; 500; 1000 µg/mL) durante 3, 12 y 24h. Ambos biomarcadores mostraron que los efectos tóxicos más evidentes aparecieron a la concentración y tiempo más altos ensayados, reduciéndose la viabilidad en un 40%, sin embargo en concentraciones y tiempos más bajos se mantenía aún un 70% de viabilidad celular. Del mismo modo, otros autores evaluaron la citotoxicidad de la arcilla comercial sin modificar, CNa+. Baek et al., 2012, evaluó mediante los mismos biomarcadores la citotoxicidad de la línea celular intestinal INT-407 expuesta a la arcilla (0- 1000 µg/mL) durante 24, 48 y 72h. Mediante el ensayo MTT se observó una inhibición de la proliferación celular después de los tres tiempos de exposición y de

forma creciente en torno a los 100µg/mL, sin embargo se aprecia un aumento de LDH únicamente en la concentración más alta ensayada después de 48 y 72h.

Además de la arcilla comercial CNa+, existen otras arcillas modificadas disponibles comercialmente, como es el caso de Cloisite®30B (C30B), Cloisite®93A (C93A), Cloisite®20A (C20A), entre otras. De las mismas también se han llevado a cabo una evaluación toxicológica así como estudios de comparación entre unas arcillas y otras. Sharma y col., 2010, evaluaron mediante el ensayo Azul Trypan (Binderup y col., 2002) la proliferación celular de la línea intestinal de adenocarcinoma de colon Caco-2 expuesta a nueve concentraciones de CNa+ y C30B (0- 226 µg/mL) durante 24h. En el caso de CNa+, no se obtuvieron diferencias significativas con respecto al control en ninguno de los casos estudiados, mientras que tras la exposición a C30B no filtrada se observa un 40% de citotoxicidad en la concentración más alta ensayada, eliminando esta concentración para futuros ensayos. En el caso de C30B filtrada se obtuvieron niveles citotóxicos menores según el biomarcador utilizado. Otros autores, más recientemente, han evaluado las mismas arcillas en la misma línea celular. Maisanaba y col., 2013a, expusieron Caco-2 a CNa+ (0- 250 µg/mL) y C30B (0- 125 µg/mL), determinando las concentraciones de exposición según su límite de dispersión en el medio, durante 24 y 48 h. En este caso se evaluaron tres biomarcadores como son el ensayo de Rojo Neutro (RN) (Borenfreud y Puerner., 1984) (Fig. 4) para evaluar el daño lisosomal, la capacidad de reducción de la sal de tetrazolio MTS (MTS) (Baltrop y col., 1991) y la proliferación celular y contenido proteico total (PT) mediante el método de Bradford (1976). En el caso de Caco-2 expuesta a CNa+ se observaron diferencias significativas con respecto al control en la concentración y tiempo más altos ensayados en el caso del biomarcador MTS, coincidiendo con los resultados de los autores nombrados anteriormente. Sin embargo, con C30B los tres biomarcadores proporcionan evidencias de citotoxicidad por exposición de la misma. En el caso del ensayo de PT, tras 24 horas de exposición se aprecian diferencias con respecto al control desde concentraciones medias ensayadas (31,25 µg/mL), sin embargo, esta citotoxicidad es más pronunciada tras 48h donde a partir de 15,65 µg/mL se observaron diferencias significativas con respecto al grupo control. Algo similar ocurre en el ensayo de RN, donde se aprecia un descenso de la viabilidad desde 62,5 y 31,25 µg/mL de exposición tras 24 y 48h, respectivamente. En la misma línea se ensayó un tercer biomarcador,

MTS, observándose tras 24h un descenso de la viabilidad a partir de 62,5 µg/mL y un descenso dependiente de concentración tras las 48 horas de exposición. El biomarcador más sensible en dicho estudio resultó ser PT, con una concentración efectiva media (CE50) de 40 µg/mL. En este caso, se obtuvieron efectos citotóxicos a concentraciones más bajas comparados con los resultados que hasta el momento había en la bibliografía.

También existen estudios llevados a cabo en otras líneas celulares que cabe destacar, con las arcillas comerciales ya comentadas. El efecto citotóxico de CNa+ también se ha evaluado en la línea celular HepG2, junto con la exposición a esta misma línea de C93A (Lordan y col., 2011). La línea celular fue expuesta a diferentes concentraciones que iban desde 1 a 1000 µg/mL, valorándose los biomarcadores MTT y liberación de la LDH tras 24h. En el caso del primer biomarcador mencionado se aprecian diferencias significativas en el rango completo de concentraciones ensayadas, siendo más acusado en la más alta, pudiéndose observar un 23 y 37% de reducción en la viabilidad y alteración mitocondrial tras la exposición de CNa+ y C93A, respectivamente. Respecto a la liberación de LDH, la línea celular HepG2 expuesta a CNa+ presentó un leve pero significativo incremento en las concentraciones de 50, 100 y 500µg/mL, mientras que a la concentración más alta ensayada no se observaron cambios de la LDH comparado con el grupo control. Sin embargo, no ocurrió lo mismo en el caso de C93A, donde se mostró una liberación de la enzima mucho más pronunciada, con una significación de $p<0,01$, desde 50-1000 µg/mL. En esta misma línea celular, Maisanaba y col., 2013b evaluó la citotoxicidad de CNa+ y C30B. El tiempo de exposición fue de 24 y 48h y el rango de concentraciones ensayadas para este caso fue para CNa+ de 0-62,5 µg/mL y para C30B de 0-500 µg/mL. Los resultados obtenidos por exposición de HepG2 a CNa+ teniendo en cuenta los mismos biomarcadores que en el caso de la línea Caco-2, mostraron que no se producía un descenso en la viabilidad en ninguno de los biomarcadores estudiados. Sin embargo, tras la exposición a C30B la línea celular sí presentó alteraciones. En el caso de PT, se observó tras 24h de ensayo cambios con respecto al grupo control en las dos concentraciones más altas ensayadas (250 y 500 µg/mL), mientras que después de 48h estas diferencias aparecen en las tres últimas concentraciones (125-500 µg/mL). El biomarcador RN coincidió en efecto en los tiempos ensayados, donde se observaron alteraciones de 62,5 a 500µg/mL en ambos periodos. Y por último, MTS denotó

diferencias en las tres concentraciones más elevadas tras 24h y de 62,5 a 500 µg/mL después de 48h de ensayo. En tal caso, la CE50 calculada fue 88µg/mL, siendo el biomarcador RN el más sensible.

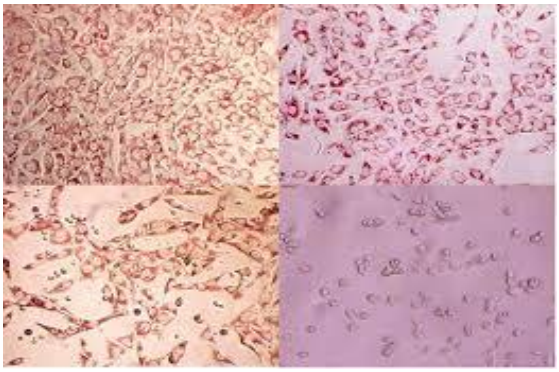

Figura 4. Vista al microscopio óptico (40x) de la captación lisosomal de Rojo Neutro de diferentes líneas celulares.

Además de todas las arcillas que hoy por hoy hay disponibles comercialmente, también encontramos algunas que se encuentran en fase de desarrollo y evaluación, como es el caso de Clay1 y Clay2. Ambas arcillas han sido desarrolladas en el Instituto Tecnológico del Embalaje, Transporte y Logística de Valencia (ITENE). Previamente al lanzamiento comercial se ha evaluado su potencial toxicólogico, estudio que se recoge en el trabajo publicado por Houtman y col., 2013, en el cual no sólo se evalúan estas dos arcillas sino también la ya comercial antes mencionada C20A.

En el caso de la evaluación de la citotoxicidad de C20A (0-62,5µg/mL) y Clay1 (0-8 µg/mL) no se observó una reducción en la viabilidad celular en ninguna de las líneas celulares, Caco-2 y HepG2, tiempos (24 y 48h) y concentraciones ensayadas. Por otro lado, sí es importante mencionar el efecto que causó Clay2 sobre las dos líneas seleccionadas. Las concentraciones ensayadas oscilaron en un rango de 0 a 125µg/mL y los biomarcadores ensayados fueron PT y MTS durante 24 y 48h de exposición. Para Caco2 se observó en el biomarcador PT un descenso en la viabilidad dependiente del tiempo y la concentración de exposición, sin embargo HepG2 presentó únicamente diferencias a 24h a 125µg/mL y a 48h en las dos últimas concentraciones. Respecto al segundo biomarcador, la línea celular Caco2 presentó diferencias significativas con respecto al control en las dos concentraciones más altas ensayadas después de 24h, presentándose aún una reducción de la actividad mitocondrial más significativa a las

48h, donde se aprecian cambios desde concentraciones medias (30µg/mL). En el caso de HepG2, ésta resulto ser de nuevo menos sensible que la anterior, observándose alteraciones en la concentración más alta tras 24h y desde 40µg/mL a 48h. En el caso de las CE50 halladas para cada una de las líneas, se obtuvieron 34 µg/mL para Caco-2 y 88 µg/mL para HepG2.

En resumen, cada arcilla, puede presentar un perfil toxicológico diferente teniendo en cuenta las líneas celulares expuestas, tiempos ensayados y concentraciones de exposición, obteniendo de tal forma una amplia diversidad en los resultados.

2.2. Ensayos de Genotoxicidad

Según la Autoridad Europea de Seguridad Alimentaria (EFSA, 2011), los ensayos de genotoxicidad son un requisito indispensable a llevar a cabo en la evaluación de toxicidad previa a la aprobación y comercialización de un compuesto destinado a entrar en contacto con alimentos. Los ensayos de genotoxicidad están diseñados para detectar compuestos que inducen directa o indirectamente daño en el material genético por diferentes mecanismos. Generalmente se considera que este daño es esencial para producir efectos heredables o para mediar las distintas etapas de formación de tumores. Existe una amplia gama de ensayos de genotoxicidad, entre ellos los más comunes para evaluar el potencial genotóxico de las arcillas son:

- Ensayo Cometa (o Single Cell Gel Electroforesis Assay): mediante una electroforesis se puede saber el daño en el núcleo celular que ha ejercido el compuesto objeto de estudio. Puede ser aplicado a cualquier línea celular y permite el análisis del daño genético a nivel de células individuales, observándose la desintegración de la hebra de ADN (Fig.5a)

- Test de Ames: considerado también como test de mutagenicidad. *Salmonella Typhimurium his*$^-$ es expuesta a las diferentes concentraciones del compuesto tóxico en un medio his$^-$. Se comprueba el crecimiento de diferentes cepas después de la exposición del tóxico, evaluando si se ha producido el crecimiento de la misma por alteración en el material genético, revirtiendo así la mutación que presenta (Fig.5b).

- Micronúcleos (MN): Los MN se producen por irregularidades en el proceso de división celular, generándose estas estructuras derivadas del núcleo, más pequeñas y que pueden contener o bien cromosomas enteros o bien fragmentos cromosómicos derivados de roturas no reparadas (Fig. 5c).

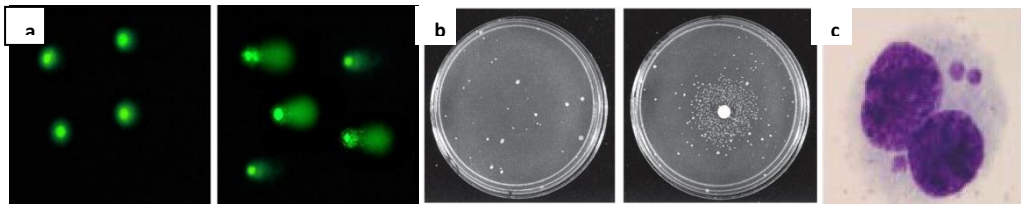

Figura 5. Visualización del resultado del Ensayo Cometa (a), Test de Ames (b) y Micronúcleos (c).

Es posible encontrar en la bibliografía científica diversos ensayos de genotoxicidad llevados a cabo con arcillas modificadas. Li y col., 2010, evaluaron la genotoxicidad en la línea CHO de NSP mediante los tres ensayos nombrados anteriormente, el ensayo Cometa, el Test de Ames y MN. En ninguno de los tres ensayos se observaron daños a las concentraciones ensayadas, concluyendo la ausencia de genotoxicidad y mutagenicidad del compuesto.

Respecto a arcillas comerciales, Sharma y col., 2010, evaluó el potencial genotóxico sobre Caco-2 de C30B, filtrada y no filtrada, mediante el ensayo Cometa, y el potencial mutagénico mediante el Test de Ames. Los resultados observados con el primer ensayo y C30B mostraron que la arcilla tanto no filtrada como filtrada resulta ser genotóxica sobre la línea celular dependiente de la concentración de exposición, presentándose las diferencias significativas más claras en las dos concentraciones más elevadas ensayadas, 113 y 170µg/mL. Otros autores también evaluaron el potencial genotóxico de C30B en Caco-2 y HepG2 mediante el ensayo Cometa (Maisanaba y col., 2013a, Maisanaba y col., 2013b). Caco-2 no presentó diferencias con el control en las concentraciones ensayadas (10, 20, 40µg/mL) a ningún tiempo de exposición (24y 48h), mientras que C30B resultó ser genotóxica para HepG2 a la concentración más alta ensayada, 88µg/mL, tras 48h de exposición.

En el caso de Clay2, ésta causó daño en el material genético de las células Caco-2 a la concentración más alta ensayada después de 24 y 48h de exposición, en el ensayo Cometa. Por otro lado, HepG2 presentó daños después de 24h expuesta a la CE50 antes mencionada, y a 48h desde la CE50/2 (Houtman y col., 2013).

2.3. Ensayos de Estrés oxidativo

La producción de formas activas de oxígeno es tóxica para los organismos y un exceso de las mismas da lugar a lo que se conoce como estrés oxidativo. La estructura electrónica del oxígeno hace que sea susceptible de reducciones parciales, dando lugar a especies reactivas de oxígeno (EROs). Las EROs se producen normalmente en las células como producto del metabolismo celular. La concentración de EROS está controlada por acción de los antioxidantes. Si un agente estresante induce un aumento en la producción de EROs, este equilibrio puede romperse causándose daños en las estructuras celulares. Los daños serán reparados por mecanismos celulares específicos. Si no se recupera el equilibrio se producirán disfunciones que pueden desembocar en la muerte celular.

Los autores hasta ahora mencionados han valorado también el estrés oxidativo como posible mecanismo de acción tóxica de las arcillas sobre las líneas celulares de estudio. Baek y col., 2012 evaluaron la posible producción de EROs en la línea INT-407. Obtuvieron diferencias significativas a una concentración aproximada de 50µg/mL después de 48 y 72h de tratamiento, mientras que a la concentración más alta ensayada fue únicamente donde se observó daño oxidativo con respecto al control en todos los tiempo ensayados. En el caso de CNa+ y C30B, Sharma y col., 2010 no encontraron daño oxidativo en Caco-2 en el rango de concentraciones ensayadas, con lo que determinaron que la genotoxicidad que producía C30B no era mediada por la producción de EROs. Sin embargo, estos últimos resultados contrastan con los encontrados por Maisanaba y col., 2013a, donde observaron tanto un aumento en la producción de EROs y diferencias en el contenido de glutatión (GSH) a 40µg/mL a ambos tiempos de exposición y al más alto ensayado, respectivamente.

Por otro lado, en los ensayos realizados con HepG2 expuesta a CNa+ y C93A se observó producción de EROs en el caso de CNa+ a partir de 50µg/mL en los tres tiempos ensayados por estos autores, mientras que los resultados obtenidos por exposición a C93A fueron más variables. Se mostró una producción de daño oxidativo en la concentración de 50µg/mL a las 24h del ensayo, y a las dos concentraciones superiores a ésta a 16 y 24h de exposición. Sin embargo, llama la atención la ausencia

de producción de EROs a la concentración más alta ensayada (Lordan y col., 2011). Estos autores sugieren a partir de los resultados obtenidos que el incremento de la generación de EROs está asociado con la citotoxicidad y el daño en la membrana celular inducido por ambas arcillas. Maisanaba y col., 2013b, no observaron diferencias con respecto al control en la producción de EROs por exposición de HepG2 a CNa+, aunque sí con el contenido en GSH, donde observaron alteraciones en el rango de concentraciones ensayadas.

Por último, los ensayos llevados a cabo por Houtman et al., 2013, para dilucidar si se producía daño oxidativo en Caco-2 y HepG2 por exposición a Clay 2, resultaron ser negativos en producción de EROs y GSH para Caco-2 en las tres concentraciones de tratamiento, mientras que para HepG2 coincidía la ausencia de EROs pero no la de GSH, mostrándose diferencias con respecto al control en todas las concentraciones de exposición.

3. Ensayos de toxicidad *in vivo*

In vivo se refiere a experimentación hecha dentro o en el tejido vivo de un organismo vivo. Los ensayos llevados a cabo *in vivo* presentan aquellas ventajas que para los *in vitro* son carencias. Éstos dan una información más completa de lo que ocurre en el organismo cuando se encuentra expuesto a cualquier xenobiótico objeto de estudio, proporcionando datos extrapolables al ser humano y pudiendo detectar efectos secundarios. Son métodos validados formalmente y son los que comúnmente se llevan a cabo para los ensayos clínicos y registro de nuevos fármacos.

Los ensayos *in vivo* llevados a cabo con arcillas son más escasos que los publicados *in vitro*, y la mayoría se centran en cortos periodos de exposición. A continuación se presenta una breve revisión de lo que actualmente se puede encontrar en la literatura científica.

3.1. Efectos a corto plazo

Autores ya mencionados en este capítulo han llevado a cabo estudios de toxicidad oral evaluando diferentes parámetros tras exponer a los roedores a concentraciones mucho más elevadas que las usadas *in vitro*. Li y col., 2010, diseñaron un experimento con cuatro grupos bien diferenciados de ratas, administrándole a cada uno una dosis única de NSP (0, 1500, 3000 y 5700 mg/mL) disuelta en agua destilada. Los animales fueron observados durante 14 días, no produciéndose mortalidad por exposición de ninguna de las dosis, ni tampoco aparición de signos clínicos ni cambios macroscópicos notables con respecto al grupo control. No hubo pérdidas de peso ni diferencias en el consumo de comida entre los grupos experimentales. Por otro lado, Baek y col., 2012, llevaron a cabo a rasgos generales el mismo ensayo que los autores anteriores pero con MMT pura y cuatro dosis diferentes (5, 50, 300 y 1000 mg/mL). Los resultados obtenidos coincidieron con Li y col., 2010, no observando ninguna anormalidad ni pérdida de peso en los animales durante el periodo de tratamiento.

Mascolo y col., 2004 realizaron un estudio en ratas centrado en la ingestión y distribución de arcillas en los diferentes órganos. Los animales estuvieron expuestos durante tres días a una dosis de 450 mg/mL de arcillas y a las 72h de la administración fueron sacrificados y procedieron a evaluar los diferentes tejidos. No se observaron efectos macroscópicos, aunque sí un aumento de la concentración de los elementos traza en varios órganos: riñón > hígado > corazón > cerebro. La captación de las arcillas estaba directamente correlacionada con la composición de las mismas dando lugar a un apreciable incremento en determinados órganos, incluso siendo destacable los elementos traza. Estos autores concluyeron que la salud de los consumidores podría verse comprometida.

También se han llevado a cabo estudios del efecto protector de la MMT, como es el caso de Abbès y col., 2007. En este estudio evalúan la capacidad de la arcilla de captar cadmio (Cd), un destacado elemento que puede causar múltiples alteraciones en el organismo. Entre otros parámetros evaluaron marcadores bioquímicos del suero sanguíneo, observando en los animales tratados con MMT (tres grupos: 400,600 y 800 mg/kg) diferencias con respecto al grupo control que estaba expuesto directamente a

$CdCl_2$. Los animales expuestos a $CdCl_2$ revelaron un incremento de los niveles en sangre de transaminasas (ALT, AST) y de LDH, y un significativo descenso de los niveles de creatinina, creatinkinasa (CK), triglicéridos y proteínas totales. Sin embargo, en los animales tratados con la arcilla se apreció una restauración de los niveles sanguíneos normales en ratas de ALT, AST y LDH comparado con el grupo control y una mejora de CK y creatinina.

3.2. Efectos a largo plazo

La escasez de ensayos de toxicidad subcrónica con arcillas modificadas es manifiesta en la bibliografía científica, a pesar de que, tal y como se ha comentado, el escenario de exposición humana más probable se corresponde con ingestas bajas y prolongadas en el tiempo. Hasta el momento podemos encontrar los llevados a cabo por Maisanaba y col., 2013c,d.

En el primero mencionado, Maisanaba y col., 2013c, se lleva a cabo un ensayo de 90 días con ratas Wistar expuestas a una dosis de 40mg/kg de Clay1, considerando la misma como escenario de peor exposición humana. Se evaluaron diferentes parámetros como fueron: la histopatología de nueve órganos (cerebro, corazón, pulmón, hígado, intestino, bazo, riñón y testículos), marcadores bioquímicos del suero sanguíneo (glucosa, urea, creatinina, PT, albúmina, colesterol, AST, ALT, fosfatasa alcalina, Na y K), la liberación de IL-6 y el ratio en sangre de GSH/ GSSG. En ninguno de los parámetros evaluados durante el estudio se experimentó una alteración significativa en ratas expuestas a Clay1 comparadas con el grupo control.

El mismo grupo evaluó la peroxidación lipídica (LPO) y la actividad de las enzimas antioxidantes Superóxido Dismutada (SOD) y Catalasa (CAT) en bazo de ratas expuestas a Clay1 (Maisanaba y col., 2013d). Los resultados obtenidos mostraron que no se produjeron diferencias con respecto al grupo control en ninguno de los tres parámetros evaluados.

4. Conclusión

A lo largo de esta revisión se han mostrado las diferentes alternativas que se presentan para poder evaluar la toxicidad de estos nuevos materiales incorporados al envasado en la industria alimentaria, las nanoarcillas. De los ensayos *in vitro* realizados hasta el momento, se puede concluir que el efecto que produzcan en este caso las nanoarcillas va a variar enormemente según la línea celular seleccionada y a la arcilla a la que se exponga (es decir, al modificador que contengan), los biomarcadores y tiempos ensayados, así como de las concentraciones seleccionadas para el tratamiento. Respecto a los ensayos *in vivo*, los escasos estudios existentes parecen indicar un bajo riesgo tóxico por exposición a estas sustancias. No obstante, serían necesarios más estudios para confirmar su seguridad.

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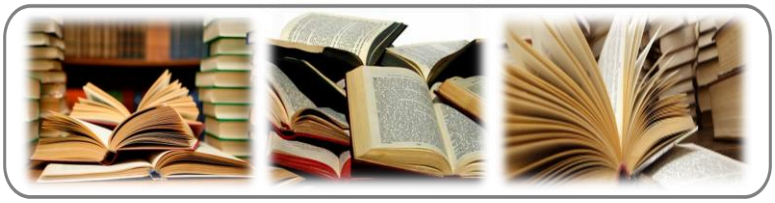

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