TESIS DOCTORAL

BÚSQUEDA DE SUSTANCIAS NUTRACÉUTICAS CONTENIDAS EN LA DIETA MEDITERRÁNEA: ANÁLISIS ANTIGENOTOXICOLÓGICO, TUMORICIDA, DE ANTIENVEJECIMIENTO Y DE MARCAS EPIGENÉTICAS.

Zahira Noemí Fernández Bedmar

2015

TITULO: *Búsqueda de sustancias nutracéuticas contenidas en la dieta mediterránea: análisis antigenotoxicológico, tumoricida, de antienvejecimiento y de marcas epigenéticas*

AUTOR: *Zahira Noemí Fernández Bedmar*

© Edita: Servicio de Publicaciones de la Universidad de Córdoba. 2016
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

UNIVERSIDAD DE CÓRDOBA

TESIS DOCTORAL

BÚSQUEDA DE SUSTANCIAS NUTRACÉUTICAS CONTENIDAS EN LA DIETA MEDITERRÁNEA: ANÁLISIS ANTIGENOTOXICOLÓGICO, TUMORICIDA, DE ANTIENVEJECIMIENTO Y DE MARCAS EPIGENÉTICAS.

Trabajo realizado en el Departamento de Genética de la Universidad de Córdoba para optar al grado de Doctor en Biociencias y Ciencias Agroalimentarias por la Licenciada en Biología:

ZAHIRA NOEMÍ FERNÁNDEZ BEDMAR

Dirigido por:

Dra. Ángeles Alonso Moraga **Dr. Joaquín Pérez-Guisado Rosa**

TÍTULO DE LA TESIS:

BÚSQUEDA DE SUSTANCIAS NUTRACÉUTICAS CONTENIDAS EN LA DIETA MEDITERRÁNEA: ANÁLISIS ANTIGENOTOXICOLÓGICO, TUMORICIDA, DE ANTIENVEJECIMIENTO Y DE MARCAS EPIGENÉTICAS.

DOCTORANDA: ZAHIRA NOEMÍ FERNÁNDEZ BEDMAR

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

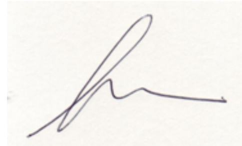
La Tesis Doctoral de Dñª. Zahira Noemí Fernández Bedmar se ha llevado a cabo en el Departamento de Genética de la Universidad de Córdoba. Su desarrollo se ha ajustado a los plazos inicialmente previstos y ha permitido a la doctoranda adquirir una sólida formación en diversas disciplinas de la Genética aplicándolas de un modo transversal. Durante la realización de la Tesis, Dñª. Zahira Noemí Fernández Bedmar ha confirmado una excelente aptitud y vocación por la investigación científica y ha demostrado poseer una excelente capacidad de trabajo, organización e integración así como de redacción de trabajos científicos. La doctoranda ha conseguido otorgar un valor añadido a ciertos alimentos contenidos en la dieta mediterránea como el pimiento rojo, la naranja, el ajo y la hesperidina, asignándoles papeles importantes en la lucha contra procesos degenerativos como: la seguridad, la protección genómica, la influencia en la longevidad y la quimioprevención. La potencia antioxidante y el papel en la modulación de la metilación de secuencias repetitivas de amplias regiones genómicas, se encuentran entre los mecanismos básicos para la potencialidad de actividad quimiopreventiva y anticarcinogénica de ciertas moléculas como la hesperidina. Derivado de su Tesis se ha publicado el primer capítulo titulado “Role of Citrus Juices and its Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity and Longevity Scopes”, en *Journal of Toxicology and Environmental Health, Part A*: Current Issues.

DOI:10.1080/15287394.2011.582306. Zahira Fernández-Bedmar, Jaouad Anter, Silvia de La Cruz-Ares, Andrés Muñoz-Serrano, Ángeles Alonso-Moraga, Joaquín Pérez-Guisado. Así mismo, está preparando cuatro artículos más.

Por todo ello, autorizamos la presentación de la Tesis Doctoral.

Córdoba, 23 de Noviembre de 2015

Firma de los directores



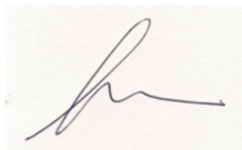
Fdo.: Ángeles Alonso Moraga **Fdo.: Joaquín Pérez-Guisado Rosa**

Dra. Ángeles Alonso Moraga y **Dr. Joaquín Pérez-Guisado Rosa**

INFORMAN:

Que el trabajo titulado **“BÚSQUEDA DE SUSTANCIAS NUTRACÉUTICAS CONTENIDAS EN LA DIETA MEDITERRÁNEA: ANÁLISIS ANTIGENOTOXICOLÓGICO, TUMORICIDA, DE ANTIENVEJECIMIENTO Y DE MARCAS EPIGENÉTICAS”** realizado por Dñª Zahira Noemí Fernández Bedmar bajo la dirección de la Dra. Ángeles Alonso Moraga y el Dr. Joaquín Pérez-Guisado Rosa, puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Firmado en Córdoba, a 23 de Noviembre de 2015



Fdo. Ángeles Alonso Moraga

Fdo. Joaquín Pérez-Guisado Rosa

Nota: esta Tesis Doctoral se presenta en parte en inglés, ya que el primer capítulo que la conforma ha sido publicado y el resto de capítulos presentes en la misma son artículos en preparación con formato de revista de investigación para ser publicados. Los objetivos específicos que se persiguen en la Tesis se desarrollan en cada uno de los capítulos, ya que se han organizado por grupos taxonómicos de alimentos. Por esta misma razón, y para facilitar la lectura, las referencias de la Introducción, Capítulos y Discusión General aparecen al final de cada uno de ellos.

ÍNDICE

Contents

AGRADECIMIENTOS ………………………………………………………………………….1

ABREVIATURAS ……………………………………………………………………………….5

RESUMEN ………………………………………………………………………………………11

SUMMARY …………………………………………………………………………………....17

INTRODUCCIÓN …………………………………………………………………………….23

1. Evolución humana, dieta y salud. ………………………………………………….25
2. Sustancias vegetales contenidas en la dieta mediterránea. …………….26
3. Alimentos no procesados presentes en la base de los platos mediterráneos ………………………………………………………………………….…..27
4. Estrés oxidativo y su relación con mecanismos genotóxicos (fundamentalmente mutagénicos) y no genotóxicos (epigenéticos) ..30
5. Modelos de ensayos para la detección de sustancias saludables …….32

HIPÓTESIS ……………………………………………………………………………………..41

OBJETIVOS……………………………………………………………………………………..45

CAPÍTULO I: Role of *Citrus* Juices and its Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity and Longevity Scopes…………………………………………………….51

CAPÍTULO II: *In vivo* and *in vitro* evaluation for nutraceutical purposes of capsaicin, capsanthin, lutein and four pepper varieties. ………………………..85

CAPÍTULO III: Biological Activities of Two *Allium* species and Their Distinctive Organosulphur Compounds. ……………………………………………….135

CAPÍTULO IV: New targets on the biological activities of lycopene and tomato………………………………………………………………………………………….177

CAPÍTULO V: Anticarcinogenic and demethylating potency of Hesperidin. *In vitro* and *in vivo* assays. ……………………………………………………………….211

DISCUSIÓN GENERAL ……………………………………………………………………..247

CONCLUSIONES GENERALES ………………………………………………………….271

GENERAL CONCLUSIONS ………………………………………………………………..275

ANEXO: PUBLICACIÓN DEL CAPÍTULO I ………………………………………….279

AGRADECIMIENTOS

Qué difícil es agradecer con palabras a tod@s los que me habéis acompañado durante esta etapa de mi vida. Los que me conocéis, sabéis que soy más de hechos que de palabras, por ello, perdón si no transmito al 100% todo lo que os quiero decir.

Gracias en primer lugar a MI FAMILIA. A **mi padre** por ser mi talismán, por transmitirme que la sabiduría no es proporcional al número de títulos, por crear en mi la inquietud de conocer, de saber….cuánto te echo de menos¡¡ … aunque físicamente no estés … siempre estarás en mí. A **mi madre** por transmitirme que la lucha y el esfuerzo diario es necesario para conseguir lo que uno quiere y se propone, por soportar mis cambios de humor, por hacer que me levantara cuando ni tan siquiera tenía fuerzas para hacerlo, por apoyarme siempre con todo esto ….. simplemente gracias por creer en mí. A mis hermanos **Raquel** y **Andrés**….MIL GRACIAS¡¡¡ de una forma u otra estáis ahí siempre y me ayudáis y a mi hermana **Virtudes** … porque en poco tiempo me transmitiste muchos valores …..gracias simplemente por ser mi referente.

A **Jesús**, mi pareja, por tener tanta paciencia conmigo, por intentar comprender todo esto, por estar ahí siempre a mi lado… y como no….a mi "bichito" **Selene**, mi hija, mi vida, mi razón de ser, gracias por ser el motor que hace que arranque con las pilas bien cargadas, por dibujar en mí una sonrisa en las situaciones más difíciles. Gracias a los dos por aguantar mis ausencias físicas y mentales y a pesar de todo ello…..quererme.

Gracias a **Angelines**, mi "mami2" por ser como es…, por "adoptarme", por brindarme su confianza y darme la oportunidad de trabajar con ella, por

esperarme siempre que he necesitado tiempo, por estar ahí en los momentos más difíciles….porque ahí estabas siempre…escuchándome, apoyándome y aconsejándome, por decirme las cosas tal cual, con sinceridad, tanto para regañarme como para felicitarme. GRACIAS TESORILLO¡¡¡¡.

Gracias a **Andrés Muñoz**, por sus charlas y por las risas durante el ratito del té, por transmitirme sus conocimientos y por ofrecerme su amistad y ayuda siempre y cuando lo necesitaba.

Gracias también a **Joaquín**, mi director de tesis, pues sin su ayuda y empeño este proyecto no hubiera sido posible.

A todos **mis amigos** y **compañeros** del Departamento, en especial a Deivid, Fernandiviri y MarySun, gracias por las risas que echamos juntos y por demostrarme lo que realmente es la amistad. Y como no… gracias a Tania y Marcos por brindarme ayuda siempre que lo he necesitado en la segunda etapa por el lab y por hacer que me sintiera bien entre ellos.

Por último gracias a todos aquellos que por olvido no les he citado………

………porque ésta, mi Tesis, es también vuestra.

ABREVIATURAS

Abreviatura	Descripción
4QNO	4-Nitrosoquinoline-Oxide
A172	Human glioblastoma cell
AFB1	aflatoxin B1
AGS	Gastric adenocarcinoma cell
ARPE-19	Arising Retinal Pigment Epithelial 19
AsPC-1	Pancreatic cáncer ascites metastasis
B16-F10	mouse melanoma cell line
BaP	benzo[*a*]pyrene
Bd^S	*Beaded Serrate*
BPDE	(+)-anti-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene
BxPC-3	Pancreatic cáncer cell line
CaCo-2	Human colon cáncer cell line
CE 81T/VGH	Esophagus epidermoid carcinoma cell line
COLO 205	Human colon adenocarcinoma cell line
COX	Cyclooxygenase
CP	cyclophosphamide
DADS	Diallyl disulfide
DAS	Diallyl sulfide
DATS	Diallyl trisulfide
DEN	n-nitrosodiethylamine
DMBA	7,12 dimethylbenz[a]anthracene
DMN	Dimethyl nitrosamine
DMS-114	Small cell lung cancer cell line
DMSO	Dimethyl sulfoxide
DPDS	Dipropyl disulfide
DPS	Dipropyl sulfide
DU-145	Human prostate carcinoma cell line
EBV-EA	Epstein-Barr virus early antigen
EROs	Especies reactivas de oxígeno
flr	*flare*
GHP	Green hot pepper
GSP	Green sweet pepper
HCT116	Human colon tumour 116
HCT-15	Human colon tumour 15
HeLa	Human cervical cancer cells

Hep2	Hepatoma 2
Hep3B	hepatoma 3B
HepG2	hepatoma G2
HL60	Human leukaemia 60
HSC-2	Human squamous cell carcinoma cell line
HSC-3	Human squamous cell carcinoma cell line
HSG	Immortalized human submandibular gland cell line
HT-29	Human colon carcinoma cell line
IC_{50}	50 % inhibition concentration
IP	Inhibition percentage
IQ	2-amino-3-methylimidazo [4,5-f] quinolone
K562	Human chronic myelogenous leukemia cell line
KU812	Leukaemic cell line
L	Large single spots
LJ	Lemon juice
LNC_aP	Human prostate cancder cell line
LOX	Lysyl oxidase
MCF-7	Human breast adenocarcinoma cell line
MCF-7 ER+	Positive estrogen receptor human breast adenocarcinoma cell line
MDA-MB-231	Human breast cáncer cell line
MDA-MB-435 ER-	Negative Estrogen receptor MDA-MB-435 breast cancer cells
MMP	Matrix metalloproteinases
MNNG	N-methyl-N´-nitro-N-nitrosoguanidine
MTCCA	1-mehtyl,1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid
mwh	Multiple wing hair
NB-4	Leukaemic cell line
NDMA	N-nitrosodimethylamine
NPYR	N-Nitrosopyrrolidine
OJ	Orange juice
PBS	Fetal bovine serum
PC-3	Human prostate cáncer cell line
PZ-HPV- 7	Normal epithelial prostate cancer cell line
R	Recombinogenic activity
RBL-2H3	Rat basophile leukaemia cell line
RHP	Red hot pepper
ROS	Reactive Oxygen Species
RSP	Red sweet pepper

RT	Room temperature
S	Small single spots
SBR	sequencing batch reactor
SCGE	Single-cell gel electrophoresis
SHSY-5Y	Human neuroblastoma cell line
SK-Hep-1	Human hepatocarcinoma cell
SK-MEL5	Human melanoma cell line
SMART	Somatic Mutation and Recombination test
SNU-1	Human gastric cancer cell line
SO	styrene oxide
SW-480	Human colon cancer cell line
T	Twin spots
TA100	*Salmonella typhymurium* 100 strain
TA102	*Salmonella typhymurium* 102 strain
TA1535	*Salmonella typhymurium* 1535 strain
TA1537	*Salmonella typhymurium* 1537 strain
TA97	*Salmonella typhymurium* 97 strain
TA98	*Salmonella typhymurium* 98 strain
TM	Tail Moment
TM3	Third Chromosome multiple inversion
U937	Leukaemic cell line
UF-1	Leukaemic cell line

RESUMEN

La producción del sector hortofrutícola se ve incrementada cada año y, paralelamente, la sociedad actual demanda productos agrícolas de calidad. Esta calidad debe ser medida no sólo en un mejor sabor sino en sus potenciales efectos saludables.

La dieta mediterránea es una dieta altamente valorada a nivel mundial debido a su alto contenido en antioxidantes naturales presentes en las matrices alimenticias que la constituyen como son las frutas y verduras entre otras. Esta dieta es muy similar a la dieta de nuestros antepasados paleolíticos y numerosos estudios multidisciplinares han mostrado los efectos positivos de esta dieta frente a enfermedades degenerativas como el cáncer, obesidad, diabetes y enfermedades cardiovasculares; además se le ha atribuido una asociación directa con la longevidad.

En el presente trabajo se han seleccionado cuatro variedades de pimiento, cebolla, ajo, naranja, limón y tomate, así como las moléculas que los caracterizan capsaicina, capsantina, luteína, DPDS, DADS, hesperidina, limoneno y licopeno.

Para determinar la calidad de dichos alimentos y comprobar si el efecto de los mismos se debe a las moléculas más distintivas embebidas en su matriz, se han llevado a cabo los siguientes estudios *in vivo* e *in vitro*:

1. Estudios *in vivo* utilizando el modelo genético de experimentación animal *Drosophila melanogaster*:
    a. Evaluación de la inocuidad o seguridad genómica.

   b. Evaluación de los efectos antigenotóxicos o protectores frente al daño genético causado por la genotoxina modelo peróxido de hidrógeno.
   c. Evaluación de la longevidad asociada a la calidad de vida.
2. Estudios *in vitro* utilizando la línea celular humana modelo de inhibición del crecimiento tumoral HL60:
   a. Evaluación del efecto citotóxico o potencial quimiopreventivo.
   b. Evaluación del efecto proapoptótico y actividad clastogénica.
   c. Evaluación de la modulación metilación global del ADN en células de leucemia tratadas con una molécula seleccionada.
3. Experiencia piloto sobre la actividad anticarcinogénica de una molécula de elección (hesperidina) en un modelo de hepatocarcinogénesis en ratas inducida por dietil nitrosamina.

Todos los alimentos y moléculas estudiadas son seguros en el ensayo de mutaciones y recombinaciones somáticas (SMART) de *Drosophila melanogaster* excepto altas dosis de pimiento verde picante, limón, capsaicina y limoneno.

Todos los alimentos y moléculas estudiadas mostraron un efecto protector frente al daño genético inducido por el peróxido de hidrógeno excepto el pimiento verde picante a altas dosis.

De todas las sustancias estudiadas únicamente el pimiento rojo dulce tiene un efecto positivo sobre la longevidad asociada con la calidad de vida en *Drosophila*, junto con las concentraciones bajas de naranja, hesperidina y limoneno.

Todos los alimentos y moléculas seleccionados tienen un efecto citotóxico frente a la lineal tumoral HL60 de leucemia humana excepto luteína, cebolla y licopeno que no llegaron a alcanzar la concentración inhibitoria 50.

Las variedades de pimiento estudiadas, ajo y limón así como capsaicina, DADS y limoneno fueron las únicas en mostrar una actividad proapoptótica en la línea tumoral HL60 induciendo fragmentación internucleosómica del ADN. Además ajo, cebolla y tomate así como sus moléculas más representativas (DADS, DPDS y licopeno) mostraron actividad clastogénica del ADN en la misma línea tumoral.

La molécula de elección hesperidina es capaz de ejercer desmetilación genómica en las secuencias repetitivas LINE-1 y ALU-M2. La experiencia piloto de hepatocarcinogénesis inducida en ratas con dietil nitrosamina es muy prometedora ya que la hesperidina induce una inhibición de los nódulos hepáticos originados por la dietil nitrosamina.

Basándonos en los resultados obtenidos en la presente Tesis, podemos concluir que no todos los componentes estudiados de la dieta mediterránea confieren el mismo nivel de protección del genoma de *Drosophila* ni son quimiopreventivos frente a células de leucemia. Además, el efecto de dosis es determinante en las actividades biológicas

encontradas en las sustancias estudiadas. Entre todas las muestras estudiadas los mejores candidatos para ser considerados como nutracéuticos o alimentos funcionales son las variedades dulces de pimiento, la naranja, el ajo y el tomate.

SUMMARY

Horticultural sector production is increasing every year and, in parallel, the current society demands quality agricultural products. This quality should be measured not only as a better taste but in their potential health effect.

Mediterranean diet is a highly valued worldwide diet due to its high natural antioxidant content present in the food matrixes such as fruits and vegetables among other. This diet is very similar to the diet of our Palaeolithic ancestors and numerous multidisciplinary studies have demonstrated the positive effects of this diet against degenerative diseases such as cancer, obesity, diabetes and cardiovascular disease; it has also been attributed a direct association with longevity.

In the present study, four pepper varieties, onion, garlic, orange, lemon and tomato, as well as the characteristic molecules present in them, capsaicin, capsanthin, lutein, DPDS, DADS, hesperidin, limonene and lycopene have been selected.

In order to determine the quality of these foods and to confirm if the effect thereof is due to the most distinctive molecules embedded in their matrixes, it has carried out the next *in vitro* and *in vivo* studies:

1. *In vivo* studies using the genetic animal model *Drosophila melanogaster*:
    a. Safety evaluation or genetic security.
    b. Evaluation of the antigenotoxic or protector effects against H_2O_2 model genotoxine -induced genetic damage.
    c. Evaluation of the longevity associated to the healthspan.
2. *In vitro* studies using the human HL60 cell line as tumour inhibition growth model:

a. Evaluation of the cytotoxic effect or chemopreventive potencial.
b. Evaluation of the proapoptotic effect and clastogenic activity.
c. Evaluation of the DNA global methyl modulation in the leukaemia cells treated with a selected molecule.

3. A pilot experience testing the anticarcinogenic activity of a choice molecule (hesperidin) using a diethyl nitrosamine-induced hepatocarcinogenesis model in rats.

All tested foods and molecules are safe in the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* except high doses of green hot pepper, lemon, capsaicin and limonene.

All tested foods and molecules showed a protector effect against H_2O_2-induced genetic damage except high doses of green hot pepper.

Only red sweet pepper, among all tested substances, has a positive effect on the longevity associated to a healthspan increase in *Drosophila*, together with low concentrations of orange, hesperidin and limonene.

All selected foods and molecules have a cytotoxic effect against the human leukaemia HL60 tumour cell line except lutein, onion and lycopene that did not achieve the 50% inhibition concentration.

The tested pepper varieties, garlic and lemon as well as capsaicin, DADS and limonene were the only samples that show a proapoptotic activity in the HL60 tumoural cell line inducing a DNA internucleosomic fragmentation. Furthermore, garlic, onion and tomato as well as their most

representative molecules (DADS, DPDS and lycopene) showed DNA clastogenic activity in the same tumoural cell line.

The selected molecule hesperidin is able to induce genomic demethylation in the LINE-1 and ALU-M2 repetitive sequences. The hepatocarcinogenic pilot experience induced in rats with diethyl nitrosamine is very promising since hesperidin induces an inhibition of liver nodules caused by diethyl nitrosamine.

Based on the results obtained in the present Thesis, we can conclude that not all studied components of the Mediterranean diet confer the same protection level to the *Drosophila* genome nor are chemopreventive against leukaemia cells. Furthermore, the dose-effect is critical for the biological activities found in the tested substances. Among all the tested samples the best candidates to be considered as nutraceutical or functional foods are sweet pepper varieties, orange, garlic and tomato.

INTRODUCCIÓN

1. Evolución humana, dieta y salud.

Continuas evidencias antropológicas indican que la dieta que consumían nuestros antepasados humanos (australopitecos) se caracterizaba por la ausencia de carbohidratos refinados, niveles elevados de fibra y proteínas y niveles comparables a los actuales de grasas insaturadas y colesterol (Konner and Eaton, 2010). Esta dieta ancestral ha contribuido a la selección de nuestra composición genética y por lo tanto hay que tener en cuenta su influencia en esos momentos evolutivos. El Neolítico parece haber tenido una mínima influencia en nuestro genoma, si comparamos ese periodo con los 2,8 millones de años de evolución que lo anteceden y que forman parte del Paleolítico. Nuestros antepasados vivían en una sociedad de cazadores y las proteínas representaban aproximadamente el 19-35%, las grasas el 28-47% y los glúcidos el 22-40% del total de calorías ingeridas (Mann, 2004). Sin embargo, la dieta humana ha cambiado drásticamente: la ingesta de proteínas ha sido reducida al 10-15%; el consumo de glúcidos ha aumentado al 45-60% a través de una mayor ingesta de cereales y productos derivados del almidón, en lugar de verduras; el consumo de grasas poliinsaturadas se ha reducido y el de la grasa saturada ha aumentado. En tan corto período evolutivo de tiempo asociado al sedentarismo y a la superpoblación, los seres humanos han sido capaces de adaptarse para sobrevivir a este gran cambio alimenticio y han colonizado la tierra, sin embargo nuestro genoma evolucionó para adaptarse a unas condiciones que hoy en día no existen y los cambios ambientales han sido muy rápidos y los genes sufren un desfase de adaptación que posiblemente sea el causante de la aparición de enfermedades crónicas en la sociedad

actual (Pérez-Guisado and Muñoz-Serrano, 2011). De hecho, Eaton y col., apuntan que esta imposible y drástica adaptación genética junto a factores como el sedentarismo y la exposición a tóxicos medioambientales son en gran medida los responsables de la obesidad, diabetes tipo 2, hipertensión arterioesclerosis y varios tipos de cáncer, entre otras enfermedades degenerativas (Eaton et al., 2010).

2. Sustancias vegetales contenidas en la dieta mediterránea.

La dieta mediterránea ha sido descrita a través de una pirámide alimenticia consistente en el consumo diario de frutas, verduras, cereales y productos no refinados y productos lácteos bajos en grasa, consumo semanal de pescado, aves de corral, patatas, aceitunas, legumbres y frutos secos y raramente dulces y huevos, consumo mensual de carne roja y productos cárnicos y consumo moderado de vino (Panagiotakos and Polychronopoulos, 2005). Los diferentes componentes de sus constituyentes son: grasas monoinsaturadas (aceite de oliva y frutos secos), ácidos grasos poliinsaturados omega-3 (grasa de pescado, verdura, frutos secos, aceites vegetales), antioxidantes como la vitamina C y E y flavonoides (frutas, verduras, vino, aceite de oliva) y fibra (cereales y hortalizas) (Mackenbach, 2007). Numerosas investigaciones han demostrado las propiedades saludables de esta dieta asociándola con la longevidad (Shahar and Itamar Grotto, 2006; Trichopoulou et al., 2003) y con una menor predisposición al padecimiento de enfermedades cardiacas, ciertos tipos de cáncer, diabetes y obesidad (Agarwal and Rao, 2000; Estruch et al., 2013; La Vecchia, 2004; Salas-Salvadó et al., 2011) . Estas propiedades saludables son debidas a su alto contenido en antioxidantes

naturales (Irigaray et al., 2007; Oh et al., 2005). Aunque al tratarse de una dieta que varía según el país mediterráneo, resulta difícil detectar cuáles son los componentes más saludables dentro de la misma. Es de destacar que este tipo de dieta es similar a la que compartían nuestros antepasados con la excepción de que en ella se incluyen alimentos procesados como lo son el aceite de oliva, el vino y el pan. Los alimentos procesados podrían conducirnos a la pérdida de una dieta saludable ya que el hombre incluye en su procesamiento aditivos y/o conservantes que podrían ser perjudiciales para la salud (Sasaki et al., 2002; Yahagi et al., 1974). Desde este punto de vista, es importante llevar a cabo una evaluación de las actividades biológicas de los alimentos contenidos en la dieta mediterránea que no sufren modificaciones ni por inclusión de aditivos ni por su proceso de obtención con el objetivo de volver a ponerlos en valor.

3. Alimentos no procesados presentes en la base de los platos mediterráneos.

Existen claras evidencias sobre la relación entre el tipo de dieta y el padecimiento o ausencia de ciertos tipos de enfermedades degenerativas como el cáncer (Rodriguez-Casado, 2014). La dieta mediterránea está basada en el consumo de fruta, verdura y aceite de oliva, siendo estos componentes una elevada fuente de antioxidantes naturales, los cuales pueden ejercer un efecto pleiotrópico protegiendo al ADN del daño genético a través del secuestro de especies reactivas de oxígeno. Estos xenobióticos están asociados a su vez con procesos tales como el envejecimiento y cáncer, en los que además interviene el control de las

marcas epigenéticas (Berghe, 2012; Kampa et al., 2009; Saura-Calixto and Goñi, 2006; Si and Liu, 2014).

Por ello, es necesario determinar a nivel molecular la seguridad de su consumo, y los mecanismos por los cuales ejercen un efecto protector del ADN, así como quimiopreventivo y regulador de los patrones epigenéticos de dichos componentes (hortalizas, vino, aceite de oliva y zumos) y de sustancias activas y distintivas presentes en ellos (triacilgliceroles, organosulfurados y fenoles de diversos grupos químicos).

De entre los alimentos básicos no procesados (frescos y que no sufren ningún tratamiento higienizante, también llamados de 1ª gama) presentes en la cocina mediterránea hemos seleccionado un grupo cuya producción mundial alcanzó un total de 361.123.625 toneladas en el año 2013 (tomates: 163.963.770 toneladas; cebollas: 85.795.191 toneladas; naranjas: 71.445.353 toneladas; ajos: 24.255.303 toneladas; limones: 15.191.482 toneladas y pimientos: 472.526 toneladas) según la FAO 2015 (Food and Agriculture Organisation of the United Nations).

El tomate, debido a su versatilidad, color y sabor, se ha posicionado como elemento clave dentro de una gran variedad de platos. Sus propiedades saludables son bien conocidas. Estudios epidemiológicos han demostrado la relación directa del consumo de tomate con la prevención de enfermedades cardiovasculares y cáncer. Un meta-análisis de estudios observacionales llevados a cabo hasta el año 2003 demostró cómo elevadas ingestas de tomate crudo y no cocinado tenían una relación inversa con el cáncer de próstata (Wei and Giovannucci, 2012). Es

interesante conocer si es el licopeno el responsable de tales propiedades beneficiosas o lo es el fruto por sí mismo al tratarse de un reservorio de gran cantidad de componentes antioxidantes que podrían actuar de manera sinérgica.

La cebolla y al ajo han sido usados durante milenios en la medicina tradicional de muchas culturas en el tratamiento de desórdenes cardiovasculares entre otros. Podríamos decir que la cebolla es un tónico natural cuyo consumo es recomendable para el ser humano pero existen pocos estudios que nos muestren la acción quimiopreventiva de la misma, a pesar de ser un alimento tan extendido a nivel mundial. Con respecto al ajo, estudios epidemiológicos sobre la relación existente entre su consumo y su efecto protector frente a enfermedades como el cáncer son controvertidos (Chiavarini et al., 2015). Al tratarse de alimentos con tan elevada producción mundial (la cebolla tres veces más que el ajo), sería conveniente tratar de elucidar qué dosis son las apropiadas para el consumo y de esta manera aportar datos sobre el consumo saludable de estos dos vegetales.

Los cítricos, entre ellos la naranja y el limón, son frutos propios de climas tipo mediterráneo y subtropical. Todo son bondades sobre sus efectos saludables puesto que previenen de procesos degenerativos tales como diabetes, enfermedades cardiovasculares o determinados tipos de cáncer (González-Molina et al., 2010). De hecho, Song and Bae (2013) tras realizar un estudio observacional a través de una revisión sistemática comprobaron que el consumo de cítricos está asociado a una reducción de cáncer de mama. Debido al importante papel que se les ha atribuido en la prevención

de enfermedades tan características y de elevada índole epidemiológica, es interesante conocer los efectos de las moléculas que los distinguen y sobre todo el efecto que pudieran tener las dosis de las mismas.

Los pimientos son un grupo de frutos herbáceos que incluyen más de 200 variedades. Hay que tener en cuenta que características tales como la pungencia y la madurez influyen en la calidad del plato donde se integren y también pueden influir en la salud. La molécula responsable de la sensación de pique/quemazón de este fruto es la capsaicina (Barceloux, 2009). Es difícil establecer la seguridad del consumo de pimientos picantes, puesto que los estudios llevados a cabo han mostrado datos controvertidos (Bode and Dong, 2011). Aunque estudios epidemiológicos apoyan que el consumo de pimientos picantes está estrechamente ligado al padecimiento de cáncer de estómago y garganta (López-Carnllo et al., 1994; Serra et al., 2002). Son conocidas las propiedades anticarcinogénicas de los frutos rojos. (Tahergorabi et al., 2015), siendo la capsantina uno de los mayores carotenoides responsables del color rojo en los pimientos (Topuz and Ozdemir, 2007). Este pigmento natural es de gran atención a nivel mundial ya que se utiliza en la industria alimentaria y cosmética y es metabolizado rápidamente en el cuerpo. Estudios epidemiológicos muestran que este carotenoide tiene un efecto inhibitorio en el cáncer de colon (Shah et al., 2014). Por lo que alimentos ricos en capsantina serían útiles para mantener un estado óptimo de salud.

4. Estrés oxidativo y su relación con mecanismos genotóxicos (fundamentalmente mutagénicos) y no genotóxicos (epigenéticos)

Las especies reactivas de oxígeno (EROs) exógenas procedentes de xenobióticos, compuestos clorados, ciertos metales, radiaciones e incluso de ciertos alimentos (Alejandre-Durán et al., 1987), así como las endógenas, causan daño celular a nivel de ADN, ARN o proteínas. Cuando se producen en exceso pueden originar una gran proporción de radicales de tipo oxidativo que se deben considerar en el cómputo final del daño genético (Klaunig and Kamendulis, 2004).

Las EROs pueden ser causantes de iniciaciones tumorales (originando lesiones en el ADN que causan transversiones, deleciones, roturas de cadenas y aberraciones cromosómicas) y también pueden modular las siguientes fases del proceso carcinogénico: la proliferación (en el ciclo celular o en el proceso de muerte celular), o la metástasis (Halliwell, 2008). En general, pueden tener un papel crucial en la modulación de procesos degenerativos (mutaciones, cáncer o envejecimiento) (Franco et al., 2008) y éstos parecen estar relacionados con cambios originados en las secuencias génicas y/o en fases transcripcionales (metilaciones o desmetilaciones de genes supresores de tumores o de oncogenes respectivamente) (Fuks, 2005).

Las alteraciones producidas por radicales oxidativos en el ADN pueden interferir con la capacidad del mismo para actuar como sustrato de las metilasas, provocando una hipometilación global de los genomas. Por ejemplo, los Rayos X, ultravioleta, gamma, o los derivados 8-hidroxi-2-desoxiguanosina originados por el ataque al ADN de radicales hidroxilo, pueden inhibir la metilación de las citosinas adyacentes en las secuencias GpC u originar metilaciones específicas (Hepburn et al., 1991).

Ya que el daño oxidativo del ADN puede afectar a la metilación conduciendo a expresión génica aberrante y posiblemente conduciendo al desarrollo de tumores, la modulación en los patrones de metilación podría usarse como marcador en la biomonitorización del proceso carcinogénico/anticarcinogénico

El estrés oxidativo celular es, por tanto, un evento que debe ser evitado o paliado debido a sus efectos pleiotrópicos, en especial aquellos que afectan al genoma.

5. Modelos de ensayos para la detección de sustancias saludables.

Ensayo de detección de mutaciones y recombinaciones somáticas en discos imaginales alares de *Drosophila melanogaster* (Graf et al., 1984). Basado en la detección de alteraciones genéticas producidas en células de discos imaginales alares de la larva, que pueden distinguirse fenotípicamente en el tejido adulto después de la expansión clonal y la metamorfosis. Nuestro grupo ha usado este ensayo para detectar actividad genotóxica en compuestos de estructura química variada, desde mutágenos a promutágenos con diferentes métodos de acción genotóxica, como agentes alquilantes, intercalantes o formadores de aductos, tanto sólidos, como líquidos, gaseosos, simples o mezclas complejas (Fernández-Bedmar et al., 2011; Moraga and Graf, 1989; Rojas-Molina et al., 2005).

Ensayo de longevidad. Es bien conocido que el proceso de envejecimiento está determinado por el estrés oxidativo. Por este motivo, es necesario detectar moléculas antioxidantes incluidas en los alimentos consumidos a diario. *Drosophila melanogaster* es un modelo genético animal utilizado en

estudios de envejecimiento puesto que tiene similares vías metabólicas y sistemas orgánicos análogos a humanos que controlan la ingesta, almacenamiento y metabolismo de alimentos (Anh et al., 2011; Baker and Thummel, 2007). Existen estudios previos en los que se ha podido demostrar cómo una dieta suplementada con vegetales incrementa la expansión de vida en *Drosophila melanogaster (Bahadorani and Hilliker, 2008; Zhang et al., 2014; Zhao et al., 2008)*.

Ensayos de inhibición del crecimiento tumoral. Las células tumorales crecidas *in vitro* como líneas inmortales son una excelente herramienta para estudiar los mecanismos de citotoxicidad o inhibición del crecimiento tumoral. Son ensayos capaces de detectar actividades enzimáticas en relación con el estrés oxidativo. Otros procesos celulares que pueden ser considerados con estos ensayos de citotoxicidad están relacionados con la integridad de los compartimentos celulares, vías de óxido-reducción, vías de transducción de señales, inducción de la apoptosis y necrosis, interfiriendo con el metabolismo celular normal y la replicación del ADN (Andreoli et al., 2003). Existen abundantes referencias sobre los efectos beneficiosos de sustancias vegetales que aluden a su protección frente al cáncer debido al efecto citotóxico de moléculas antioxidantes presentes en ellas, como por ejemplo el limoneno en células de leucemia humana HL60 (Fernández-Bedmar et al., 2011).

Ensayos de determinación de la vía de muerte celular (apoptosis o necrosis). Un complemento a los ensayos de viabilidad celular consiste en detectar si la toxicidad observada es debida a una muerte celular programada o apoptosis. Ésta se caracteriza por la activación de caspasas y

por cambios morfológicos y bioquímicos celulares (Budihardjo et al., 1999; Hengartner, 2000). Estos cambios por lo general implican: contracción celular, condensación de la cromatina, formación de cuerpos apoptóticos y fragmentación del ADN (Häcker, 2000). Los ensayos de fragmentación del ADN en geles convencionales de agarosa para detectar el efecto proapoptótico de una substancia implican la aparición de un patrón característico de bandas cuyos fragmentos de moléculas de ADN internucleosómico son de 180-200 pb o múltiplos de éstos (Fernández-Bedmar et al., 2011). Las roturas de doble y simple cadena de ADN se pueden detectar no bioquímicamente sino con técnicas citogenéticas, pudiendo determinarse el nivel de daño genético ejercido incluso en una célula individual. Estos ensayos denominados del cometa (por la estela de ADN que se visualiza en electroforesis de células individuales tratadas) complementan la información de fragmentación internucleosomal para determinar la posible vía apoptótica (Olive and Banáth, 2006).

Ensayos de Biomonitorización del status de metilación global del ADN. Estudios realizados con gemelos monocigóticos han podido evidenciar cómo los factores ambientales (tabaco, actividad física y dieta entre otros), influyen en la modulación de marcas epigenéticas (Fraga et al., 2005). Debido a que los procesos epigenéticos son dinámicos, reversibles y susceptibles a factores exógenos, éstos ofrecen la oportunidad de quimioprevención o intervención a través de la dieta por vía epigenética (Chen and Xu, 2010). Además estudios preclínicos y clínicos sugieren que parte de los efectos preventivos del cáncer están asociados con alimentos bioactivos relacionados con patrones de metilación del ADN (Davis and

Uthus, 2004).

REFERENCIAS

Agarwal, S., Rao, A.V., 2000. Tomato lycopene and its role in human health and chronic diseases. Can. Med. Assoc. J. 163, 739-744.

Alejandre-Durán, E., Alonso-Moraga, A., Pueyo, C., 1987. Implication of active oxygen species in the direct-acting mutagenicity of tea. Mutat. Res. 188, 251-257.

Andreoli, C., Gigante, D., Nunziata, A., 2003. A review of in vitro methods to assess the biological activity of tobacco smoke with the aim of reducing the toxicity of smoke. Toxicol. in Vitro 17, 587-594.

Anh, N.T.T., Nishitani, M., Harada, S., Yamaguchi, M., Kamei, K., 2011. A Drosophila model for the screening of bioavailable NADPH oxidase inhibitors and antioxidants. Mol. Cell Biochem. 352, 91-98.

Bahadorani, S., Hilliker, A.J., 2008. Cocoa confers life span extension in Drosophila melanogaster. Nutr. Res. 28, 377-382.

Baker, K.D., Thummel, C.S., 2007. Diabetic larvae and obese flies—emerging studies of metabolism in Drosophila. Cell metabolism 6, 257-266.

Barceloux, D.G., 2009. Pepper and capsaicin (Capsicum and Piper species). Disease-a-Month 55, 380-390.

Berghe, W.V., 2012. Epigenetic impact of dietary polyphenols in cancer chemoprevention: lifelong remodeling of our epigenomes. Pharmacol. Res. 65, 565-576.

Bode, A.M., Dong, Z., 2011. The two faces of capsaicin. Cancer Res. 71, 2809-2814.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., Wang, X., 1999. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Bi. 15, 269-290.

Chen, J., Xu, X., 2010. Diet, epigenetic, and cancer prevention. Adv. Genet. 71, 237-255.

Chiavarini, M., Minelli, L., Fabiani, R., 2015. Garlic consumption and colorectal cancer risk in man: a systematic review and meta-analysis. Public Health Nutr., 1-10.

Davis, C.D., Uthus, E.O., 2004. DNA methylation, cancer susceptibility, and nutrient interactions. Exp. Biol. Med. 229, 988-995.

Eaton, S.B., Konner, M.J., Cordain, L., 2010. Diet-dependent acid load, Paleolithic nutrition, and evolutionary health promotion. Am. J. Clin. Nutr. 91, 295-297.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., 2013. Primary prevention of cardiovascular disease with a Mediterranean diet. New Engl. J. Med. 368, 1279-1290.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suñer, D., Cigudosa, J.C., Urioste, M., Benitez, J., 2005. Epigenetic differences arise during the lifetime of monozygotic twins. P. Natl. Acad. Sci. U.S.A. 102, 10604-10609.

Franco, R., Schoneveld, O., Georgakilas, A.G., Panayiotidis, M.I., 2008. Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett. 266, 6-11.

Fuks, F., 2005. DNA methylation and histone modifications: teaming up to silence genes. Curr. Opin. Genet. Dev. 15, 490-495.

González-Molina, E., Domínguez-Perles, R., Moreno, D., García-Viguera, C., 2010. Natural bioactive compounds of Citrus limon for food and health. J. Pharmaceut. Biomed. 51, 327-345.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Häcker, G., 2000. The morphology of apoptosis. Cell Tissue Res. 301, 5-17.

Halliwell, B., 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch. Biochem. Biophys 476, 107-112.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770-776.

Hepburn, P., Margison, G., Tisdale, M., 1991. Enzymatic methylation of cytosine in DNA is prevented by adjacent O6-methylguanine residues. J. Biol. Chem. 266, 7985-7987.

Irigaray, P., Newby, J., Clapp, R., Hardell, L., Howard, V., Montagnier, L., Epstein, S., Belpomme, D., 2007. Lifestyle-related factors and environmental agents causing cancer: an overview. Biomed. Pharmacother. 61, 640-658.

Kampa, M., Pelekanou, V., Notas, G., Castanas, E., 2009. Olive oil phenols, basic cell mechanisms and cancer. Olive Oil: Minor Constituents and Health, 129-172.

Klaunig, J.E., Kamendulis, L.M., 2004. The role of oxidative stress in carcinogenesis. Annu. Rev. Pharmacol. 44, 239-267.

Konner, M., Eaton, S.B., 2010. Paleolithic nutrition twenty-five years later. Nutr. Clin. Pract. 25, 594-602.

La Vecchia, C., 2004. Mediterranean diet and cancer. Public Health Nutr. 7, 965-968.

López-Carnllo, L., Avila, M.H., Dubrow, R., 1994. Chili pepper consumption and gastric cancer in Mexico: a case-control study. Am. J. Epidemiol. 139, 263-271.

Mackenbach, J.P., 2007. The Mediterranean diet story illustrates that "why" questions are as important as "how" questions in disease explanation. J. Clin. Epidemiol. 60, 105-109.

Mann, N., 2004. Paleolithic nutrition: what can we learn from the past? Asia Pac. J. Clin. Nutr. 13.

Moraga, A.A., Graf, U., 1989. Genotoxicity testing of antiparasitic nitrofurans in the Drosophila wing somatic mutation and recombination test. Mutagenesis 4, 105-110.

Oh, S.-Y., Lee, J.H., Jang, D.K., Heo, S.C., Kim, H.J., 2005. Relationship of nutrients and food to colorectal cancer risk in Koreans. Nutr. Res. 25, 805-813.

Olive, P.L., Banáth, J.P., 2006. The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1, 23.

Panagiotakos, D.B., Polychronopoulos, E., 2005. The role of Mediterranean diet in the epidemiology of metabolic syndrome; converting epidemiology to clinical practice. Lipids Health Dis. 4, 7.

Pérez-Guisado, J., Muñoz-Serrano, A., 2011. A pilot study of the Spanish ketogenic Mediterranean diet: an effective therapy for the metabolic syndrome. J. Med. Food 14, 681-687.

Rodriguez-Casado, A., 2014. The health potential of fruits and vegetables phytochemicals: notable examples. Crit. Rev. Food Sci., 00-00.

Rojas-Molina, M., Campos-Sánchez, J., Analla, M., Muñoz-Serrano, A., Alonso-Moraga, Á., 2005. Genotoxicity of vegetable cooking oils in the Drosophila wing spot test. Environ. Mol. Mutagen. 45, 90-95.

Salas-Salvadó, J., Bulló, M., Babio, N., Martínez-González, M.Á., Ibarrola-Jurado, N., Basora, J., Estruch, R., Covas, M.I., Corella, D., Arós, F., 2011. Reduction in the Incidence of Type 2 Diabetes With the Mediterranean Diet Results of the PREDIMED-Reus nutrition intervention randomized trial. Diabetes Care 34, 14-19.

Sasaki, Y.F., Kawaguchi, S., Kamaya, A., Ohshita, M., Kabasawa, K., Iwama, K., Taniguchi, K., Tsuda, S., 2002. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat. Res. 519, 103-119.

Saura-Calixto, F., Goñi, I., 2006. Antioxidant capacity of the Spanish Mediterranean diet. Food Chem. 94, 442-447.

Serra, I., Yamamoto, M., Calvo, A., Cavada, G., Baez, S., Endoh, K., Watanabe, H., Tajima, K., 2002. Association of chili pepper consumption, low socioeconomic status and longstanding gallstones with gallbladder cancer in a Chilean population. Int. J. Cancer 102, 407-411.

Shah, S.N.M., Tian, S.-L., Gong, Z.-H., Arisha, M.H., 2014. Studies on Metabolism of Capsanthin and Its Regulation under Different Conditions in Pepper Fruits (Capsicum spp.). Ann. Res. Rev. Biol. 4, 1106-1120.

Shahar, R., Itamar Grotto, D., 2006. Mediterranean Diet and Longevity. Curr. Nutr. Food Sci. 2.

Si, H., Liu, D., 2014. Dietary antiaging phytochemicals and mechanisms associated with prolonged survival. J. Nutr. Biochem. 25, 581-591.

Song, J.-K., Bae, J.-M., 2013. Citrus fruit intake and breast cancer risk: a quantitative systematic review. J. Breast Cancer 16, 72-76.

Tahergorabi, Z., Abedini, M.R., Mitra, M., Fard, M.H., Beydokhti, H., 2015. "Ziziphus jujuba": A red fruit with promising anticancer activities. Pharmacogn. Rev. 9, 99.

Topuz, A., Ozdemir, F., 2007. Assessment of carotenoids, capsaicinoids and ascorbic acid composition of some selected pepper cultivars (Capsicum annuum L.) grown in Turkey. J. Food Compos. Anal. 20, 596-602.

Trichopoulou, A., Costacou, T., Bamia, C., Trichopoulos, D., 2003. Adherence to a Mediterranean diet and survival in a Greek population. New Engl. J. Med. 348, 2599-2608.

Wei, M.Y., Giovannucci, E.L., 2012. Lycopene, tomato products, and prostate cancer incidence: a review and reassessment in the PSA screening era. J. Oncol. 2012.

Yahagi, T., Nagao, M., Hara, K., Matsushima, T., Sugimura, T., Bryan, G.T., 1974. Relationships between the carcinogenic and mutagenic or DNA-modifying effects of nitrofuran derivatives, including 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, a food additive. Cancer Res. 34, 2266-2273.

Zhang, Z., Han, S., Wang, H., Wang, T., 2014. Lutein extends the lifespan of Drosophila melanogaster. Arch. Gerontol. Geriat. 58, 153-159.

Zhao, T., Zhang, Q., Qi, H., Liu, X., Li, Z., 2008. Extension of life span and improvement of vitality of Drosophila melanogaster by long-term supplementation with different molecular weight polysaccharides from Porphyra haitanensis. Pharmacol. Res. 57, 67-72.

HIPÓTESIS

El hombre ha podido colonizar la Tierra gracias a la aparición de la agricultura. Todo ello a través de técnicas de cultivo que le ha permitido obtener de forma controlada vegetales y a su vez optimizar la producción y calidad de los mismos. El posterior desarrollo del comercio ha dado lugar a un empuje al crecimiento económico de los países. Por ello, la agricultura se considera una de las actividades económicas, sociales y ambientales más esenciales del ser humano.

En la actualidad, la sociedad tiene como reto el uso de la agricultura no sólo para alimentar a la humanidad sino para vivir más y mejor. En este sentido nos basaremos en el paradigma de la dieta mediterránea para intentar detectar sustancias presentes en la misma y que tengan un valor añadido al simple nutricional. De esta manera, podremos aprovechar los alimentos mejorados no sólo para producir más sino para conseguir en los mismos una calidad y propiedades saludables que nos puedan ofrecer entre otras cosas una mayor longevidad y ciertos niveles de protección frente al cáncer. Concretamente nos interesaremos en productos de origen vegetal no procesados, por lo que excluiremos del estudio aquellos productos procesados entre los que se encuentran el aceite, el vino y el pan.

En base a los criterios anteriormente mencionados, esta tesis aborda la detección de sustancias que puedan ser caracterizadas como nutracéuticas o alimentos funcionales a través del estudio de propiedades saludables de los mismos. Para ello, como cuerpo de la tesis se han seleccionado: los cítricos más característicos (naranja y limón) y las verduras que forman parte de la base de los platos típicos mediterráneos (tomate, cebolla, ajo y

pimiento). Para poder identificar las moléculas responsables de la actividad biológica de estos alimentos, se estudiarán así mismo las más distintivas contenidas en ellos.

OBJETIVOS

Objetivo general: estudiar la modulación de mecanismos genotóxicos y no genotóxicos implicados en procesos degenerativos por componentes no procesados de la dieta mediterránea y sus correspondientes compuestos activos, utilizando para ello sistemas experimentales modelo de diferente objetivo y nivel de complejidad.

Objetivos específicos:

(i) Estudiar los mecanismos de modulación de la genotoxicidad /antigenotoxicidad de las mezclas complejas así como sus sustancias simples más representativas.

(ii) Estudiar la incidencia de las mezclas complejas y sustancias simples sobre parámetros de longevidad.

(iii) Estudiar los mecanismos de inhibición del crecimiento tumoral *in vitro* de tales mezclas complejas y sustancias simples (viabilidad celular e inducción de la apoptosis).

(iv) Evaluar la modulación de marcas epigenéticas en amplias regiones genómicas por la acción de sustancias candidato de elección contenidas en la dieta mediterránea.

(v) Estudiar el potencial anticarcinogénico de una molécula de elección entre aquellas que indiquen un mayor potencial nutracéutico

Los objetivos del presente trabajo se han conseguido aplicando el siguiente **diseño experimental general**:

i. Sustancias seleccionadas: Se ha seleccionado un grupo de alimentos no procesados de uso mundial, que constituyen el cuerpo central de la cocina mediterránea: sustancias complejas (*Capsicum anuum*, *Allium sativum*, *Allium cepa*, *Lycopersicum esculentum*, *Citrus sinensis*, *Citrus limonium*) y moléculas contenidas en las anteriores (capsaicina, capsantina, luteína, DADS, DPDS, licopeno, limoneno y hesperidina). Se han utilizado controles negativos como el agua y como control positivo el peróxido de hidrógeno ya que es un modelo de toxina de tipo oxidativo.

ii. Ensayos de geno/antigenotoxicidad en el sistema eucariótico SMART. Se determinará la inocuidad a nivel de daño genético de las moléculas seleccionadas en discos imaginales alares de *Drosophila* en proliferación y además se desarrollarán ensayos de antigenotoxicidad de las que resulten no genotóxicas frente a potentes genotoxinas de tipo oxidante.

iii. Ensayos de longevidad en el sistema eucariótico modelo de *Drosophila*. Se llevarán a cabo según el método modificado por el grupo de investigación en que se incluye el proyecto de Tesis. Los ensayos de longevidad se llevarán a cabo con las mismas larvas transheterocigotas de 72 horas utilizadas en el ensayo SMART con el fin de poder hacer una comparación entre los resultados obtenidos en ambos ensayos. Las larvas de 72 ± 12 horas serán separadas en grupos de 100 individuos y el medio será suplementado con las diferentes concentraciones de las sustancias a ensayar. Los adultos emergentes serán anestesiados con CO_2 y separados en grupos de 10 individuos en viales de longevidad. Se monitorizará toda la expansión de la vida de cada individuo para cada control y concentración

establecida para finalmente hacer una estimación de las curvas de supervivencia.

iv. Ensayos de citotoxicidad en la línea modelo de leucemia humana HL60. Las curvas de inhibición del crecimiento tumoral se obtendrán mediante el método de exclusión del azul tripán midiendo la supervivencia a las 72 h en condiciones estándar de HR (80%) y CO_2 (5%) de los cultivos celulares tratados con diferentes concentraciones de las sustancias seleccionadas.

v. Determinación del nivel de inducción de apoptosis en la línea tumoral HL60. Para explicar los mecanismos de los efectos citotóxicos se estudiará la actividad proapoptótica. Se determinará la capacidad de fragmentación del ADN visualizando unidades de 180-200 pb en geles de agarosa a partir de extracciones de ADN genómico. Además se llevará a cabo el ensayo del cometa, que corroborará a nivel unicelular este proceso apoptótico. Para ello, las células HL60 serán tratadas durante 5 horas (al igual que en los ensayos de fragmentación de ADN), con las diferentes concentraciones seleccionadas. Tras el tratamiento sufrirán un proceso de lisado, alcalinización y neutralización. Tras dichos procesos el ADN celular es teñido con yoduro de propidio, y las células se visualizarán en un microscopio de fluorescencia, midiendo el parámetro Tail Moment (cuyos valores indicarán necrosis o apoptosis).

vi. Modulación de la metilación en sistemas modelo de células tumorales. Se diseñará una experiencia piloto de tratamientos con una molécula candidata y se monitorizará *in vitro* el nivel de metilación

alcanzado en amplias zonas genómicas de ADN repetitivo usando el método de modificación con bisulfito sódico y PCR específica de cadena.

vii. Ensayo de inhibición de los efectos de una carcinogénesis inducida utilizando una molécula de elección entre aquellas con potencial nutracéutico.

<u>CAPÍTULO I</u>: Role of *Citrus* Juices and its Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity and Longevity Scopes.

Chapter I

Artículo publicado en:

Journal of Toxicology and Environmental Health, Part A: Current Issues

DOI:10.1080/15287394.2011.582306

Zahira Fernández-Bedmar[a], Jaouad Anter[a], Silvia de La Cruz-Ares[a], Andrés Muñoz-Serrano[a], Ángeles Alonso-Moraga[a], Joaquín Pérez-Guisado[a]

[a]Departamento de Genética, Universidad de Córdoba, Campus Rabanales, 14071 Córdoba, Spain.

ABSTRACT

World-wide breakfast beverages content high quantities of *Citrus* juices. The purpose of the present research is to assess the nutraceutical value of orange and lemon juices as well as two of their active compounds: hesperidin and limonene. Indicator assays were performed at three levels to evaluate different biological health promoter activities: (i) determination of the safety and DNA-damage protecting ability against free radicals by using the Somatic Mutations and Recombinations Test of *Drosophila melanogaster*; (ii) study of the modulating role for life span in *Drosophila melanogaster* and (iii) measurement of the cytotoxic activity against the human tumour cell line HL60. The highest concentrations assayed for lemon juice and limonene (50 % v/v and 0.73 mM respectively) showed genotoxic activity inducing somatic recombinations. Orange and lemon juices as well as hesperidin and limonene exhibit antigenotoxic activity against hydrogen peroxide used as an oxidative genotoxine. Life span experiments revealed that the lower concentrations of the orange juice, hesperidin and limonene exerted a positive incidence on the health span of *Drosophila* when measured as the survival at the highest percentiles. Finally all the substances showed cytotoxic activity, being hesperidin the less active. Taking into account the safety, antigenotoxicity, longevity and cytotoxicity data obtained in the different assays, the orange juice can be a candidate as a nutraceutical food because it is not genotoxic, is able to protect DNA against free radicals and inhibits the tumour cell growing.

Key words: Genotoxicity, Antigenotoxicity, Cytotoxicity, Longevity, *Citrus*, hesperidin, limonene

INTRODUCTION

Inappropriate dietetic habits are estimated to be the cause of more than one third of cancer deaths. Many of these cancers could be avoided with an increased consumption of fruits and vegetables as hundred of epidemiological data suggest (Smith-Warner et al., 2006). Plant-based foods provide the organism with high content in antioxidants that could help to protect cells from the biological damage caused by free radicals that can trigger cancer development (Reddy et al., 2003). More precisely, fruit consumption has been associated to reduced risk of cancer of the upper digestive tract, stomach and urinary tract ((Vecchia and Bosetti, 2006).

Orange (OJ) and lemon (LJ) juices contain a number of benefit micronutrients (phenols, vitamin C, minerals, dietetic fibre, essential oils and carotenoids) that help to prevent degenerative processes such as diabetes, cardiovascular diseases or certain types of cancer (da Silva, 2005; González-Molina et al., 2010). The major flavonoid in sweet oranges and lemon is hesperidin (Garg et al., 2001; Gattuso et al., 2007) that is hydrolyzed by gut microflora into aglycone form (hesperetin) (Vallejo et al., 2010). Hesperidin is used in treatments against hair fragility due to its ability to reduce the permeability of the vascular endothelium. This phenol exhibits antioxidative activity via antiradical and anti-lipoperoxidation activities (Tripoli et al., 2007). It also exerts anti-inflammatory activity because it inhibits the LOX, COX and phospholipase A enzymes (Benavente-García et al., 1997) and modulates the glucose, cholesterol and fatty acid metabolisms (Jung et al., 2004; Jung et al., 2006). Hesperidin prevents bone

mass loss (Chiba et al., 2003) and can inhibit chemically induced breast cancer (So et al., 1996), bladder cancer (Yang et al., 1997), and colon cancer (Miyagi et al., 2000; Tanaka et al., 1997a; Tanaka et al., 1997b) in animal models. The distinctive flavour component in OJs and LJs is limonene. This monocyclic terpene is the major component in the *Citrus* essential oils (Crowell, 1999; González-Molina et al., 2010), used as flavour in cosmetic, beverages, foods and gums. Although mutagenicity assays showed negative results in Salmonella (Program, 1990) and in rats (Turner et al., 2001) it is considered as a non-genotoxic carcinogen (Tennant and Ashby, 1991). Some others animal experiments allow to conclude that limonene could be interesting in chemoprevention because it inhibits the tumour growing and the metastasis via apoptosis (Lu et al., 2004).

Fresh home-made *Citrus* juices are one of the most popular fruit beverages as member of so called healthy breakfasts. Therefore, it is necessary to evaluate the nutraceutical potency of a chronically consumed food through the entire life of people. Among others, several testing steps should be accomplished for a food to be health promoter: (i) the safety with respect to genetic damage; (ii) the potential protective role of DNA integrity; (iii) the influence on life span extension as a complex biological trait and (iv) the specific cytotoxic activity against transformed cells as chemopreventive agent.

The Somatic Mutation and Recombination Test (SMART) has been used in the present paper to detect mutagenic and recombinagenic activity in the clone expansion of imaginal discs of *Drosophila melanogaster* larvae. This wing spot test has been proved to be a versatile and reliable system to test

genotoxicity and antigenotoxicity of single compounds as well as complex mixtures due to the capabilities of treated larvae to bio-activate metabolites (Anter et al., 2010; Graf et al., 1994). The ability of LJ, OJ as well as two of their major components (hesperidin and limonene) to inhibit the mutagenicity induced by a model oxidative genotoxine such as hydrogen peroxide was studied. H_2O_2 causes oxidative damage on DNA by producing adducts, such as 8-hydroxy-guanine, which exert an important role in the mutagenesis process with an increase of induced transitions (Lim and Lim, 2006). Hydrogen peroxide induces also a deregulation of methylation patterns of oncogenes (Cerda and Weitzman, 1997) and the inhibition of DNA repair enzymes (Hu et al., 1995).

The expected health promoting properties of LJ, OJ and its distinctive compounds could extend the longevity in *Drosophila melanogaster*. The Life span of this insect is relatively short and the adults seem to show many of the cell senescence features as in mammals (Fleming et al., 1992). For that main reasons the fruit fly has extensively used in the study of physiological, pathological and other processes involved in life expectance, as well as to understand the relationships between food metabolism and ageing (Li et al., 2010). Average life span data of *Drosophila melanogaster* vary widely and are strongly dependent on the rearing conditions (Li et al., 2008; Mockett and Sohal, 2006; Trotta et al., 2006).

Cytotoxicity bioassays in Vitro are also needed in the assessment of the chemopreventive effects of a substance as a fast, not expensive and informative first step of screening. The human cell line HL60 provides a reliable model to study the cytotoxic effect of chemopreventive substances

and the mechanisms underlying this potential activity (Villatoro-Pulido et al., 2009). Once the cytotoxic activity of a nutraceutic is assayed, a visible test of DNA fragmentation was carried out in order to investigate whether the mechanism undergoing the cytotoxicity is mediated via apoptosis.

METHODS

Fruits and single compounds

Juices from two *Citrus* species and two single compounds were selected. Oranges (*Citrus sinensis* var. Valencia Late) and lemons (*Citrus limon* var. Lunario) were obtained in a local market. Hesperidin and limonene as single compounds contained in the fruits were purchased from Sigma and Fluka (H5254 and 62118 respectively).

Preparation of the samples

Fruits were washed with ethanol (70%) prior to the elaboration of the juice. Both OJ and LJ were prepared using a domestic manual squeezer. Fresh juices from ten fruits were mixed, aliquoted and stored at -80ºC until utilisation. In the case of cytotoxicity assays juices were centrifuged for 1 min. at 13000 rpm and the supernatant was stored at -80º. Limonene was dissolved in ethanol.

Genotoxicity and antigenotoxicity assays (SMART)

Drosophila melanogaster strains

Two *Drosophila* Strains were used, each with a hair marker in the third chromosome:

- *mwh*/*mwh*, carrying the recessive mutation mwh (**multiple wing hairs**) that produces multiple tricomas per cell (Yan et al., 2008).

- *flr*3/*In* (3LR) *TM*3, *ri pp sep bx34e es BdS*, where the *flr*3 (**flare**) marker is a homozygous recessive lethal mutation that produces deformed tricomas but it is viable in homozygous somatic cells once larvae start the development (Ren et al., 2007). See Lindsley and Zimm (2012) for more detailed information on the rest of the genetic markers.

Flies are maintained at 25ºC, 80% humidity, in a home-made meal (1000mL water, 0.5 g NaCl, 100 g yeast, 25 g sucrose, 12 g agar-agar, 5 ml propionic acid, 3.5 mL of a 0.2% sulphate streptomycin solution) and with three changes per week.

Treatments

The genotoxicity assays were carried out following the method described by Graf et al. (1984). Briefly, transheterozygous larvae for *mwh* and *flr*3 genes were obtained crossing 200 optimally virgin females (4 days old) of *flr*3 strain with 100 males of *mwh* strain. Four days after fertilisation, females were allowed to lay eggs in fresh yeast medium for 8 hours in order to obtain synchronized larvae. After 72±4 hours larvae were washed with distilled water and groups of 100 individuals were placed in the different treatments vials where a chronic treatment was followed until pupation. Treatment vials contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 mL of different concentrations of the substance to be tested. Two concentrations of each *Citrus* juice were assayed (0.75 %v/v and 50 %v/v) as well as of

hesperidin and limonene (0.0038 and 0.34 mM, 0.011 and 0.73 mM respectively). Single compounds concentrations correspond to their content in the fresh juices (Gattuso et al., 2007; Selli et al., 2004). The negative controls were prepared with medium and water and positive controls with medium and 0.15 M hydrogen peroxide (Sigma, H1009) as oxidative genotoxicant (Anter et al., 2010).

The antigenotoxicity tests were performed following the method described by Graf et al. (1998) which consisted of combined treatments of the genotoxine (0.15 M hydrogen peroxide) and the different concentrations of the juices/single compounds assayed. After emergence adult flies were stored in 70 % ethanol.

Mutations scoring

Forty transheterozygous marker wings (*mwh flr*+/*mwh*+ *flr*3) of each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400x magnification. Similar number of males and females-wings were mounted and wing hair mutations were scored among a total of 24.400 monotricoma wild-type cells per wing (Moraga and Graf, 1989). In the balancer-heterozygous genotypes (*mwh*/*TM*3, *BdS*), mwh spots phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homologue is a lethal event. To quantify the recombinagenic potency of the positive control, the frequency of *mwh* clones on the marker transhetorozygous wings (mwh single spots plus twin

spots) is compared with the frequency of mwh spots on the balancer transheterozygous wings. The difference in *mwh* clone frequency is a direct measure of the proportion of recombination (Frei et al., 1992). In the case of genotoxic results for single treatments, balancer wings (*mwh*/Bds) were also mounted in order to quantify the somatic recombinogenic activity (R) of the substance(Zordan et al., 1991) by the following formula:

R = (1 - mwh spots on the balancer wings/ mwh spots on the marker wings) X 100

Data evaluation and statistical analysis

Wing hair spots were grouped into three different categories: S, a small single spot corresponding to one or two cells exhibiting the mwh phenotype; L, a large single spot with three or more cells showing mwh or flr3 phenotypes; T, a twin spot corresponding to two juxtaposing clones, one showing the mwh phenotype and other the flr3 phenotype. Small and large spots can be originated by somatic point mutation, chromosome aberration as well as somatic recombination while twin spots are produced exclusively by somatic recombination between the *flr*3 locus and the centromere. The total number of spots was also evaluated.

A multi-decision procedure was applied to determine whether a result is positive, inconclusive or negative (Frei and Würgler, 1988; Frei and Würgler, 1995). The frequencies of each type of mutant clone per wing were compared to the concurrent negative control and the significance was given at the 5 % level. All inconclusive and positive results were

analyzed with the non-parametric U-test of Mann, Whitney and Wilcoxon ($\alpha=\beta=0.05$, one sided).

In combined treatments the inhibition of mutagenic events for juices and single compounds was calculated for total spots as proposed by Abraham (1994) by means of the following formula:

Inhibition = (genotoxine alone - sample plus genotoxine) x 100/genotoxine alone

Lifespan assays

Drosophila melanogaster strains

Animals who undergo the longevity experiments exhibited the same genotype as in genotoxicity assays in order to compare genotoxicity and longevity results. The F1 progeny from *mwh* and *flr*3 parental strains produced by an egg lying of 24 hours in yeast was used. Longevity experiments were carried out at 25ºC and following the procedure of Chavous et al. (2001). Briefly, synchronized transheterozygous larvae of 72±12 hours old were washed and separated into groups of 100 individuals in vials with a mixture of Instant Medium and 4mL of the different concentrations of the four substances selected. Emerging adults were collected, anesthetized under CO_2 and placed in 1mL longevity vials in groups of 10 individuals. Three replicates were used during the complete live extension for each control and concentration established. The survivals were counted and the medium renewed twice a week.

Statistical analysis of life span

The Kaplan–Meier estimates of the survival function for each control and concentration are plotted as survival curves. The statistical analyses and signification of the curves were assessed by the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA) using the Log-Rank (Mantel-Cox) method.

Cytotoxicity assays

Cell culture

The promyelocytic leukaemia cell line HL60 was used to assess the cytototoxic effects of juices and phenols. Cells were cultured in RPMI 1640 medium (Biowhittaker, BE12-167F), supplemented with 10% heat-inactivated bovine serum (Biowhittaker, DE14-801F), 200mM L-Glutamine (Sigma, G7513) and an antibiotic-antimycotic solution with 10000 units of penicillin, 10 mg of streptomycin and 25 µg amphotericin B per mL (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The cultures were plated at a density of 25 x 10^4cells/ml in 40 ml culture bottles (25 cm^2) and passed every two days.

Assessment of cell viability

HL60 cells were placed in 12 well culture plates (1 x 10^5cells/ml) and treated for 72 h with different concentrations of OJ, LJ, hesperidin and limonene. The cell viability was assessed utilizing the trypan blue exclusion method. Trypan blue is a vital dye, and its reactivity is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. Trypan blue (Fluka, 93595) was added to the cell culture

with a volume ratio of 1:1. The number of living cells was counted using a hemocytometer under an inverted microscope (Motic, AE30/31) at 100 X magnifications. Each experiment was repeated in triplicate, growth curves were established and IC_{50} values were estimated. Curves are plotted as survival percentage with respect to the control growing at 72 h.

Analysis of DNA fragmentation

In order to detect DNA fragmentation in cells entering apoptosis, HL60 cells ($1.5x10^6$/ml) were treated with different concentrations of the substances for 5 h. Treated cells were collected, centrifuged at 4000 rpm for 5 min. and washed with PBS. DNA was extracted using a commercial DNA extraction kit (Dominion mbl, 243), and treated with RNase before loading. A final amount of 1500 ng DNA was subjected to a 2% agarose gelelectrophoresis (50V for 2 h) and stained with ethidium bromide.

RESULTS

Genotoxicity and Antigenotoxicity testing of *Citrus* juices and component

The SMART assay was used to asses the health promoting properties of *Citrus* species and its distinctive compounds. **Table 1** shows the results for genotoxicity testing of the four substances assayed in the SMART. All the substances are non-mutagenic at the lowest concentration of the assayed rank. This lower concentration was chosen taking into account the daily food intake for a *Drosophila* larva and giving a similar juice intake to a human consumption of 250mL/day. Nevertheless lemon juice and limonene are mutagenic (0.325 spots/wing) in the SMART at the highest assayed concentration (50%v/v and 0.73mM respectively). In order to

Table 1. Genotoxicity of Lemon and Orange Juices, hesperidin and limonene in the Drosophila wing spot test.

evaluate the recombinogenic potency of mutagenic concentrations, we looked at additional information on the spots per wing scored in balancer-heterozygous wings where *mwh* clones reflect only somatic point mutations and chromosome aberrations, since somatic recombination is a lethal event. Values of recombinogenicity with respect to the total induced clones were 77 and 62.5% for lemon juice and limonene respectively.

Compounds	N	Small spots (1-2 cells)	Large spots (>2 cells)	Twin spots	Total spots
H_2O	40	0.15 (6)[a]	0	0	0.15 (6)
Lemon Juice (% v/v)					
0.75	40	0.27 (11) **ns**	0	0	0.27 (11)**ns**
50	40	0.3 (12)**ns**	0	0.02 (1)**ns**	0.32 (13)*
50 *Serrate*	40	0.07 (3)	0	0	0.07 (3)
Orange Juice (% v/v)					
0.75	40	0.22 (9)**ns**	0	0	0.22 (9)**ns**
50	40	0.15 (6)**ns**	0.02 (1)**ns**	0	0.17 (7)**ns**
Hesperidin (mM)					
0.0038	40	0.27 (11)**ns**	0	0	0.27 (11)**ns**
0.24	40	0.15 (6)**ns**	0	0	0.15 (6)**ns**
Limonene (mM)					
0.011	40	0.27 (11)**ns**	0	0	0.27 (11)**ns**
0.73	26	0.3 (12)**ns**	0.02 (1)**ns**	0	0.32 (13)*
0.73 *Serrate*	40	0.12 (5)	0	0	0.12 (5)**ns**

a: number of spots per wing, N: number of wings; ns: non-significant (p>.005), *: Statistically significant compared with the control (p≤0.05) . The data were evaluated by the non-parametric U test of Mann, Whitney and Wilcoxon according to Frei and Würgler (1995).

Table 2. Antigenotoxicity of Lemon and Orange Juices, hesperidin and limonene in the Drosophila wing spot test.

Compounds	N	Small spots (1-2 cells)	Large spots (>2 cells)	Twin spots	Total spots	IP
Controls						
Negative (H_2O)	40	0.15 (6)[a]	0	0	0.15 (6)	
Positive (H_2O_2)	40	0.4 (16)*	0.02 (1)**ns**	0.02 (1)**ns**	0.45 (18)*	
Lemon Juice (% v/v)						
0.75	40	0.32 (13) **ns**	0	0.02 (1)**ns**	0.35 (14)**ns**	22.2
50	40	0.4 (16)*	0	0	0.4 (16)*	11.1
Orange Juice (% v/v)						
0.75	40	0.25 (10)**ns**	0	0	0.25 (10)**ns**	44.4
50	40	0.1 (4)**ns**	0.12 (5)**ns**	0	0.22 (9)**ns**	50.0
Hesperidin (mM)						
0.0038	40	0.17 (7)**ns**	0.02 (1)**ns**	0	0.2 (8)**ns**	55.5
0.24	40	0.3 (12)**ns**	0	0	0.3 (12)**ns**	27.8
Limonene (mM)						
0.011	40	0.2 (8)**ns**	0	0	0.2 (8)**ns**	55.5
0.73	40	0.07 (3)**ns**	0.02 (1)**ns**	0.02 (1)**ns**	0.12 (5)**ns**	72.2

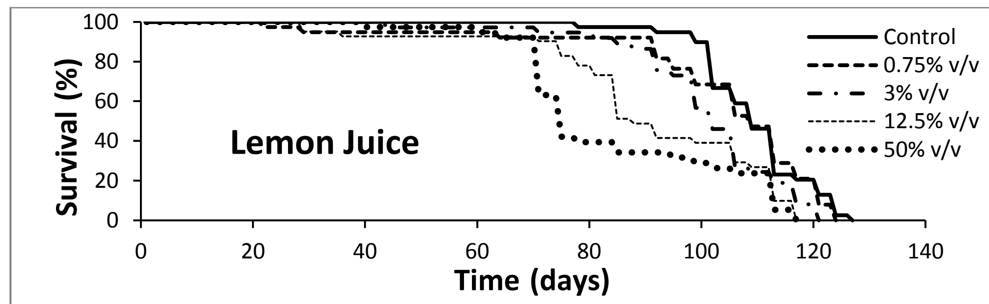
a: number of spots per wing, N: number of wings; ns: non-significant (p>.005), *: Statistically significant compared with the control ($p \leq 0.05$). IP: inhibition percentage. The data were evaluated by the non-parametric U test of Mann, Whitney and Wilcoxon according to Frei and Würgler (1995).

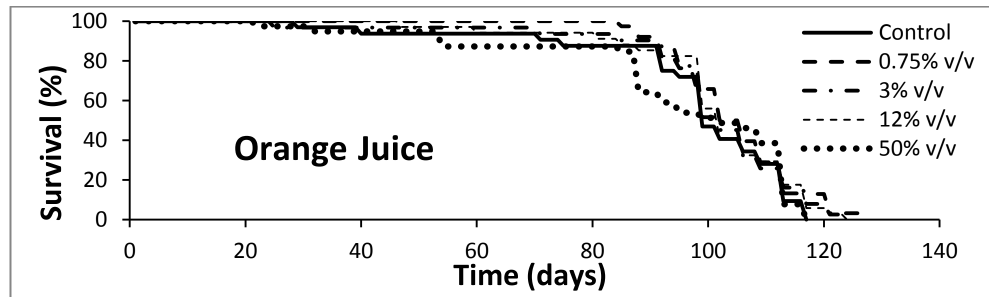
Table 2 shows the results for antigenotoxicity assays performed in the combined treatments where larvae are fed chronically with the genotoxicant hydrogen peroxide (0.15 M) and the different concentrations of the *Citrus* juices or components. Hydrogen peroxide is a well-known mutagen in *D. melanogaster* and has been used to induce microsatellite instability in mismatch repair mutants (López et al., 2002). The genotoxine hydrogen peroxide exhibited a mutation rate of 0.45 spots/wing. This result is in agreement with others obtained using the same genetic background (Anter et al., 2010; Villatoro-Pulido et al., 2009). The antigenotoxic potency of the four substances studied against hydrogen peroxide showed no clear-cut dose-response effect. Average values for the inhibition percentage of the genotoxicity of hydrogen peroxide were: 16.5, 41.6, 47 and 64 % for LJ, hesperidin, OJ and limonene respectively.

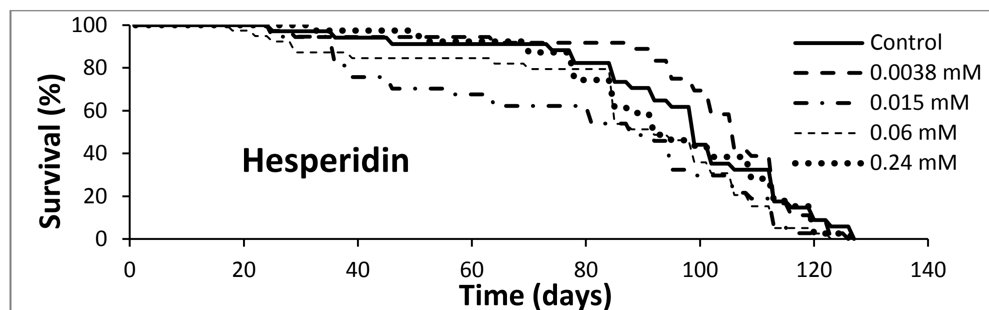
Longevity assays

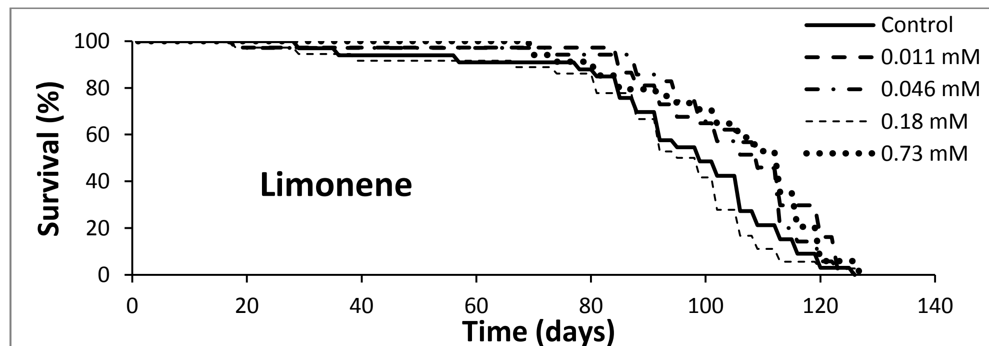
Figure 1 shows the survival curves obtained by the Kaplan-Meier method for *Drosophila melanogaster* under chronic treatments with different concentrations of LJ, OJ, hesperidin and limonene and the respective water controls. The entire life span curves were analyzed statistically by the method Log-Rank (Mantel-Cox) (data not shown). For controls, average and maximum of entire life span values were 99.2 and 123 days respectively. Log-Rank (Mantel-Cox) analyses for complete life span have shown no significant differences between treatment curves and control for orange juice. In the case of lemon juice, higher concentrations (3, 12.5 and 50%

Figure 1. Survival curves of *Drosophila melanogaster* fed with different concentrations of lemon and orange Juices, hesperidin and limonene over time.









v/v) curves were statistically different to the water-control and the lower concentration (0.75% v/v) ones with a decrease of life span. Hesperidin curves at 0.15 and 0.06 Mm were also statistically lower than the water control and the lowest concentration (0.0038mM) curves. Finally, 0.0111 and 0.18 mM limonene supplementation significantly increased the average life span compared to control flies fed with normal food.

In vitro Human Leukaemia cytotoxicity assays

A wide rank of concentrations was used for every substance (0.625-2.5%, 0.75-20%, 0.37-25mM and 0.035-2.34mM, for LJ, OJ, hesperidin and limonene respectively). The **Figure 2** shows the relative tumour growth inhibition for the substances assayed. Lemon juice presented an IC50 (1.4%) lower than orange juice (4.4%). The dose-response curves were different for the two juices exhibiting lemon juice a wide plateau for the lower concentrations. Hesperidin and limonene exerted cytotoxic effect on HL60 cells although the IC_{50} for limonene (0.2 mM) is lower than that of hesperidin (14 mM).

Figure 3 shows the electrophoresis of the genomic integrity in HL60 cells treated for 5 hours with different concentrations of the substances. DNA nucleosomal fragmentation was observed in median-highest concentrations of lemon juice (0.8, 1.2 1.4, 1.6, 1.8, and 2 % v/v) and in the three highest concentrations of limonene (0.6, 1.2 and 2.35 mM). This characteristic laddering of apoptotic activity was not observed in orange juice and hesperidin samples.

Figure 2. Cytotoxicity of lemon and orange juices, hesperidin and limonene on HL60 cells.

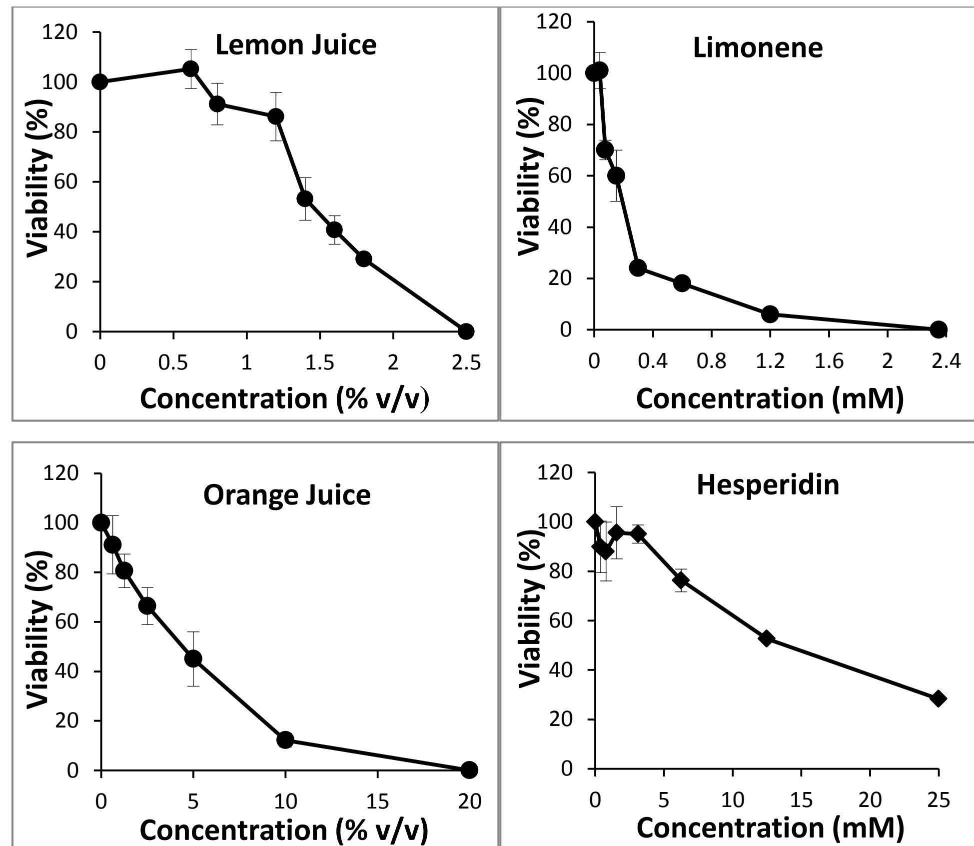
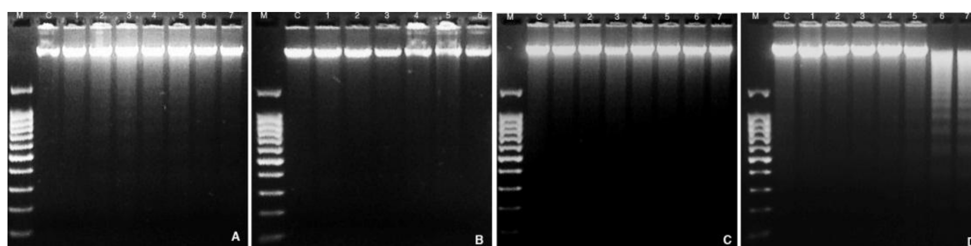


Figure 3. DNA fragmentation induced in HL60 cells by lemon and orange juices (A,B), hesperidin (C) and limonene (D). HL60 human leukemia cells were exposed for 5 h to different concentrations of tested compounds. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min.



Lemon Juice (A): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 0.8 % v/v (lane 2); 1.2 % v/v (lane 3); 1.4 % v/v (lane 4); 1.6% v/v (lane 5); 1.8 % v/v (lane 6); 2 % v/v (lane 7). Orange Juice (B): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 1.25 % v/v (lane 2); 2.5 % v/v (lane 3); 5 % v/v (lane 4); 10 % v/v (lane 5); 20 % v/v (lane 6). Hesperidin (C): marker (lane M); control (lane C); 0.39 mM (lane 1); 0.78 mM (lane 2); 1.52 mM (lane 3); 3.12 mM (lane 4); 6.25 mM (lane 5); 12.5 mM (lane 6); 25 mM (lane 7). Limonene (D): marker (lane M); control (lane C); 0.037 mM (lane 1); 0.075 mM (lane 2); 0.15 mM (lane 3); 0.3 mM (lane 4); 0.6 mM (lane 5); 1.2 mM (lane 6); 2.35 mM (lane 7).

DISCUSSION AND CONCLUSIONS

The results in the wing spot test for orange juice gave non-significant values at the assayed concentrations when compared to the water control. Mutagenicity of orange juices has been found only in the Ames Salmonella test using the TA97 and TA98 strains with and without metabolic activation (Franke et al., 2004). Contrarily, when the Swiss Webster mice eukaryotic model was used to carry out the comet assay in peripheral white blood cells, the orange juice was non-genotoxic (Franke et al., 2005). Being *Drosophila* a eukaryotic model, our results in the wing spot test are in agreement with those of the comet assay in mice. Lemon juice has been tested in the wing spot test of *Drosophila* and resulted genotoxic inducing recombinogenic activity at the higher concentration (50% v/v); our research give the first result available with respect to the genetic safety of lemon juice. Hesperidin was non-genotoxic in the somatic mutations and recombinations assay of *Drosophila melanogaster* and our results agree with the lack of genotoxicity detected in the *Salmonella* TA98 assay with or without metabolic activation by Van der Merwe et al. (2006). Limonene is not mutagenic in the Ames system using four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) (Program, 1990). Nevertheless our results indicated genotoxic activity for limonene at the higher assayed concentration in the *Drosophila* wing spot test. Our data showed that limonene could cause oxidative stress and ROS generation acting as a pro-oxidant at the highest concentration. This finding agrees with the results in eukaryotic cells that suggest that limonene could act as a pro-oxidant agent depending on the assayed concentration (Bakkali et al.,

2008). Genotoxicity results of the *Citrus* juices and component assayed suggested that the limonene content of lemon juice could be responsible for the recombinagenic activity observed in the highest concentration of lemon juice. The differential content of limonene in LJ and OJ (86 and 17 mg/L) given by Maccarone et al. (1998) would reflect such an association between genotoxicity of lemon juice and limonene at the higher concentration and the lack of genotoxicity of orange juice.

Our antigenotoxicity data for orange juice obtained in *Drosophila* against the oxidative genotoxine H_2O_2 (47% average inhibition percentage) are in agreement with those obtained by da Silva (2005) who demonstrated that orange juice could inhibit the DNA damage produced by alkylating agents in carrying the comet assay in mice. Higashimoto et al. (1998) founded a 36% mutagenicity-reducing activity of lemon juice against nitrite-treated MTCCA using the TA100 strain of *Salmonella typhimurium*; our results for lemon juice are also in agreement with the AMES test showing an average of the inhibition percentage of 16.5%. The different antigenotoxic potencies of the OJ and LJ could be related to the differential content in antioxidants. It is known that the antioxidant potency of *Citrus* is due to the ascorbic acid and phenolic contents (Gardner et al., 2000) and that orange juice contains higher β-carotene equivalents, ascorbic acid and total phenolics than lemon juice (Xu et al., 2008).

The inhibition ability of hesperidin against the genotoxic effects of hydrogen peroxide in the imaginal discs of *Drosophila* was higher at the lowest concentration (55.5%). Kalpana et al. (2009) found hesperidin radioprotective by effectively decreasing micronucleus frequency, dicentric

aberrations and comet attributes and related this activity to its ability for ROS scavenging. The higher content of hesperidin in orange juice in comparison to lemon juice, 58 and 20 mg/100mL respectively (Cano et al., 2008; Gattuso et al., 2007) would explain the antigenotoxic ability of orange juice. Limonene inhibited the genotoxicity of hydrogen peroxide, behaving as a reductor agent that would protect cells from the hydrogen peroxide oxidative stress (Hernández et al., 2007; Roberto et al., 2010).

The antiproliferative activity of orange juices has been tested in various K562 (human chronic myelogenous leukemia), HL60 (human leukemia) and MCF-7 (human breast adenocarcinoma) cell lines showing that a concentration of 10%v/v was able to inhibit 73% of HL60 cells growth (Camarda et al., 2007), being the correspondent data in our experiments to 85%. The cytotoxicity of lemon juice against HL60 cells found in the present work has also been reported for Caco-2 and HpG2 cell lines (Lim and Lim, 2006; Sun et al., 2002). The cytotoxicity of hesperidin has been assayed in different cell lines (MDA-MB-435 ER-, MCF-7 ER+, DU-145, HT-29, DMS-114, SK-MEL5) by Manthey and Guthrie (2002) showing no antiproliferative activity due to the glysosylation of the molecule moiety. Nevertheless, many in vivo researches concluded that hesperidin presents anticancer activity in lung, oral, colon and bladder carcinogenesis (Kamaraj et al., 2009; Tanaka et al., 1997a; Tanaka et al., 1997b; Yang et al., 1997); these results are in concordance with the in vitro assays of the present work performed in the HL60 cell line. With respect to the cytotoxicity of limonene, Tatman and Mo (2002) obtained a similar inhibitory concentration to that of the present paper (0.18 and 0.20 mM

respectively). In vivo assays for limonene are contradictory: it seems to inhibit the appearance of liver and gastric tumours in mice (Lu et al., 2004; Parija and Das, 2003) but Turner et al. (2001) showed limonene as chemical agent able to induce kidney and bladder tumours in male rats.

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis. The results of our study have shown fragmentation of DNA upon treatment of HL60 cells with LJ and Limonene indicating the involvement of apoptosis. A dose-dependent relationship in the treatment with lemon juice at lower concentrations was observed. At the two highest doses, it seems that cells are initiating the necrotic process, which could explain the absence of the DNA fragmentation. Limonene induced a slight DNA fragmentation at 0.6 mM. This effect was clearer at highest concentrations (1.2 and 2.35mM) resulting in a dose-dependent response. This result could be related with an initiation of the apoptotic process in the tumoural HL60 cells. Limonene seems to act like a pro-apoptotic agent with promising antitumoural properties. Rabi and Bishayee (2009) demonstrated the apoptotic effect of limonene in DU-145 prostate cancer cells but not in normal epithelial prostate PZ-HPV- 7 cells. Besides, in human colon cancer cells (SW-480) a DNA fragmentation and induction of caspase-3 by lime volatile oils has been shown, which may be due to the involvement of apoptosis mechanism (Patil et al., 2009).

We have compared the survival curves for water control and the rest of substances at ≥75% of living flies. The health span significations were as follows: Orange juice lower dose treatments of 0.75 and 3.25%v/v compared to non-enriched diet water control significantly increased health

span ($p \leq 0.05$); every lemon juice treatments decreased health span when compared to water control ($p \leq 0.01$ in all the cases), as the very low pH of lemon juice (2.3) could affect negatively and differentially *Drosophila* adults survival (Mai et al., 2010); the lower concentration of hesperidin (0.0038 mM) increased the health span ($p \leq 0.05$) and the two lowest concentrations of limonene (0.011 and 0.046 mM) also improved significantly the health span ($p \leq 0.05$). Taking into account that the maximum averages life span for ≤75% survivals are 91, 98, 95, 92, 95, 92 days for water control and the above mentioned correspondent orange, lemon, hesperidin and limonene significant concentrations, a general increase trend is observed in both mean and maximum lifespan. That means an increase of the health span portion of the life span. Taken together, this study uncovers the effects of orange and lemon juices on *Drosophila melanogaster* longevity, which results from a combination of antioxidative and prooxidative activities. Given that the fruit fly is an import model for studies on human nutrition and pharmacology, the results of this work suggest that moderate consumption of orange juice and its active and mayor components (hesperidin and limonene) may have the potential to strengthen the antioxidant defence system and, consequently, to extend their life span and increase the health span. However, considering the fact that *Citrus* juices may also exhibit pro-oxidant activities toward the mitochondria, life span extension may vary depending on genetic and environmental factors (Arking and Conn, 2005).

The results obtained in the present paper showed different aspects of the activity of LJ, OJ as well as hesperidin and limonene. Genotoxicity data

advised on the mutagenic activity of lemon juice and limonene at the highest concentrations. Antigenotoxicity assays indicated that all the genetic safe concentrations are antigenotoxic showing different inhibition percentages. All the substances exerted cytotoxic activity although only lemon juice and limonene was able to enters the DNA fragmentation as an apoptotic way. Finally, as a biological multivariate trait, life span studies suggested that the lower concentrations of orange juice, hesperidine and limonene increased the health span part of the life span curves. Orange juice as a complex mixture and hesperidin and limonene as single compounds can be proposed as substances to be studied deeply as potential nutraceuticals.

REFERENCES

Abraham, S.K., 1994. Antigenotoxicity of coffee in the Drosophila assay for somatic mutation and recombination. Mutagenesis 9, 383-386.

Anter, J., Campos-Sánchez, J., El Hamss, R., Rojas-Molina, M., Muñoz-Serrano, A., Analla, M., Alonso-Moraga, Á., 2010. Modulation of genotoxicity by extra-virgin olive oil and some of its distinctive components assessed by use of the Drosophila wing-spot test. Mutat. Res. 703, 137-142.

Arking, R., Conn, M., 2005. Gene expression and the extended longevity phenotypes of Drosophila. Handbook of models for human aging, 283-298.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils–a review. Food Chem. Toxicol. 46, 446-475.

Benavente-García, O., Castillo, J., Marin, F.R., Ortuño, A., Del Río, J.A., 1997. Uses and properties of citrus flavonoids. J. Agr. Food Chem. 45, 4505-4515.

Camarda, L., Di Stefano, V., Del Bosco, S.F., Schillaci, D., 2007. Antiproliferative activity of Citrus juices and HPLC evaluation of their flavonoid composition. Fitoterapia 78, 426-429.

Cano, A., Medina, A., Bermejo, A., 2008. Bioactive compounds in different citrus varieties. Discrimination among cultivars. J. Food Compos. Anal. 21, 377-381.

Cerda, S., Weitzman, S., 1997. Influence of oxygen radical injury on DNA methylation. Mutation Research/Reviews in Mutation Research 386, 141-152.

Crowell, P.L., 1999. Prevention and therapy of cancer by dietary monoterpenes. J. Nutr. 129, 775S-778S.

Chavous, D.A., Jackson, F.R., O'Connor, C.M., 2001. Extension of the Drosophila lifespan by overexpression of a protein repair methyltransferase. P. Natl. Acad. Sci. 98, 14814-14818.

Chiba, H., Uehara, M., Wu, J., Wang, X., Masuyama, R., Suzuki, K., Kanazawa, K., Ishimi, Y., 2003. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. J. Nutr. 133, 1892-1897.

da Silva, J., 2005. Influence of orange juice over the genotoxicity induced by alkylating agents: an in vivo analysis. Mutagenesis 20, 279-283.

Fleming, J., Reveillaud, I., Niedzwiecki, A., 1992. Role of oxidative stress in Drosophila aging. Mutat. Res. 275, 267-279.

Franke, A.A., Cooney, R.V., Henning, S.M., Custer, L.J., 2005. Bioavailability and antioxidant effects of orange juice components in humans. J. Agr. Food Chem. 53, 5170-5178.

Franke, S., Ckless, K., Silveira, J., Rubensam, G., Brendel, M., Erdtmann, B., Henriques, J., 2004. Study of antioxidant and mutagenic activity of different orange juices. Food Chem. 88, 45-55.

Frei, H., Clements, J., Howe, D., Würgler, F.E., 1992. The genotoxicity of the anti-cancer drug mitoxantrone in somatic and germ cells of Drosophila melanogaster. Mutat. Res. 279, 21-33.

Frei, H., Würgler, F., 1988. Statistical methods to decide whether mutagenicity test data from Drosophila assays indicate a positive, negative, or inconclusive result. Mutat. Res. 203, 297-308.

Frei, H., Würgler, F.E., 1995. Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in Drosophila. Mutat. Res. 334, 247-258.

Gardner, P.T., White, T.A., McPhail, D.B., Duthie, G.G., 2000. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. Food Chem. 68, 471-474.

Garg, A., Garg, S., Zaneveld, L., Singla, A., 2001. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. Phytother. Res. 15, 655-669.

Gattuso, G., Barreca, D., Gargiulli, C., Leuzzi, U., Caristi, C., 2007. Flavonoid composition of citrus juices. Molecules 12, 1641-1673.

González-Molina, E., Domínguez-Perles, R., Moreno, D., García-Viguera, C., 2010. Natural bioactive compounds of Citrus limon for food and health. J. Pharmaceut. Biomed. 51, 327-345.

Graf, U., Abraham, S.K., Guzmán-Rincón, J., Würgler, F.E., 1998. Antigenotoxicity studies in Drosophila melanogaster. Mutat. Res.- Fund. Mol. M. 402, 203-209.

Graf, U., Moraga, A.A., Castro, R., Carrillo, E.D., 1994. Genotoxicity testing of different types of beverages in the Drosophila wing somatic mutation and recombination test. Food Chem. Toxicol. 32, 423-430.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Hernández, C.E.L.R., Werberich, D.S., de Mattos, M.C.S., D'Elia, E., 2007. Electrogeneration of hydrogen peroxide applied to the peroxide-mediated oxidation of (R)-limonene in organic media. Electronic Journal of Biotechnology.

Higashimoto, M., Yamato, H., Kinouchi, T., Ohnishi, Y., 1998. Inhibitory effects of citrus fruits on the mutagenicity of 1-methyl-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid treated with nitrite in the presence of ethanol. Mutat. Res. 415, 219-226.

Hu, J.J., Dubin, N., Kurland, D., Ma, B.-L., Roush, G.C., 1995. The effects of hydrogen peroxide on DNA repair activities. Mutat. Res. 336, 193-201.

Jung, U.J., Lee, M.-K., Jeong, K.-S., Choi, M.-S., 2004. The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice. J. Nutr. 134, 2499-2503.

Jung, U.J., Lee, M.-K., Park, Y.B., Kang, M.A., Choi, M.-S., 2006. Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. Int. J. Biochem. Cell B. 38, 1134-1145.

Kalpana, K., Srinivasan, M., Menon, V.P., 2009. Evaluation of antioxidant activity of hesperidin and its protective effect on H2O2 induced oxidative damage on pBR322 DNA and RBC cellular membrane. Mol. Cell Biochem. 323, 21-29.

Kamaraj, S., Ramakrishnan, G., Anandakumar, P., Jagan, S., Devaki, T., 2009. Antioxidant and anticancer efficacy of hesperidin in benzo (a) pyrene induced lung carcinogenesis in mice. Invest. New Drugs 27, 214-222.

Li, S., Chen, K., Li, X., Zhang, X., Liu, S.V., 2010. A new cultivation system for studying chemical effects on the lifespan of the fruit fly. Exp. Gerontol. 45, 158-162.

Li, Y.M., Chan, H.Y.E., Yao, X.Q., Huang, Y., Chen, Z.Y., 2008. Green tea catechins and broccoli reduce fat-induced mortality in Drosophila melanogaster. J. Nutr. Biochem. 19, 376-383.

Lim, S.-L., Lim, L.-Y., 2006. Effects of citrus fruit juices on cytotoxicity and drug transport pathways of Caco-2 cell monolayers. Int. J. Pharmaceut. 307, 42-50.

Lindsley, D.L., Zimm, G.G., 2012. The genome of Drosophila melanogaster. Academic Press.

López, A., Xamena, N., Marcos, R., Velázquez, A., 2002. Germ cells microsatellite instability: The effect of different mutagens in a mismatch repair mutant of Drosophila (spel1). Mutat. Res. 514, 87-94.

Lu, X.-G., Zhan, L.-B., Feng, B.-A., Qu, M.-Y., Yu, L.-H., Xie, J.-H., 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. World J. Gastroentero. 10, 2140-2144.

Maccarone, E., Campisi, S., Fallico, B., Rapisarda, P., Sgarlata, R., 1998. Flavor components of Italian orange juices. J. Agr. Food Chem. 46, 2293-2298.

Mai, W.-j., Yan, J.-l., Wang, L., Zheng, Y., Xin, Y., Wang, W.-n., 2010. Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in tilapia (Oreochromis niloticus) blood cells. Aquat. Toxicol. 100, 271-281.

Manthey, J.A., Guthrie, N., 2002. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. J. Agr. Food Chem. 50, 5837-5843.

Miyagi, Y., Om, A., Chee, K., Bennink, M., 2000. Inhibition of azoxymethane-induced colon cancer by orange juice. Nutr. Cancer 36, 224-229.

Mockett, R.J., Sohal, R.S., 2006. Temperature-dependent trade-offs between longevity and fertility in the Drosophila mutant, methuselah. Exp. Gerontol. 41, 566-573.

Moraga, A.A., Graf, U., 1989. Genotoxicity testing of antiparasitic nitrofurans in the Drosophila wing somatic mutation and recombination test. Mutagenesis 4, 105-110.

Parija, T., Das, B.R., 2003. Involvement of YY1 and its correlation with c-myc in NDEA induced hepatocarcinogenesis, its prevention by d-limonene. Mol. Biol. Rep. 30, 41-46.

Patil, J.R., Jayaprakasha, G., Murthy, K.C., Tichy, S.E., Chetti, M.B., Patil, B.S., 2009. Apoptosis-mediated proliferation inhibition of human colon cancer cells by volatile principles of Citrus aurantifolia. Food Chem. 114, 1351-1358.

Program, N.T., 1990. NTP Toxicology and Carcinogenesis Studies of d-Limonene (CAS No. 5989-27-5) in F344/N Rats and B6C3F1 Mice (Gavage Studies). National Toxicology Program technical report series 347, 1.

Rabi, T., Bishayee, A., 2009. d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J. Carcinogen. 8, 9.

Reddy, L., Odhav, B., Bhoola, K., 2003. Natural products for cancer prevention: a global perspective. Pharmacol. Therapeut. 99, 1-13.

Ren, N., Charlton, J., Adler, P.N., 2007. The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. Genetics 176, 2223-2234.

Roberto, D., Micucci, P., Sebastian, T., Graciela, F., Anesini, C., 2010. Antioxidant activity of limonene on normal murine lymphocytes: relation to H2O2 modulation and cell proliferation. Basic Clin. Pharmacol. 106, 38-44.

Selli, S., Cabaroglu, T., Canbas, A., 2004. Volatile flavour components of orange juice obtained from the cv. Kozan of Turkey. J. Food Compos. Anal. 17, 789-796.

Smith-Warner, S., Genkinger, J., Giovannucci, E., 2006. Fruit and vegetable consumption and cancer. Nutr. Oncol., 97-173.

So, F.V., Guthrie, N., Chambers, A.F., Moussa, M., Carroll, K.K., 1996. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices.

Sun, J., Chu, Y.-F., Wu, X., Liu, R.H., 2002. Antioxidant and antiproliferative activities of common fruits. J. Agr. Food Chem. 50, 7449-7454.

Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Ogawa, H., 1997a. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18, 957-965.

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara, A., Sumida, T., Fukutani, K., Tanaka, T., Ogawa, H., 1997b. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. Cancer Res. 57, 246-252.

Tatman, D., Mo, H., 2002. Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells. Cancer Lett. 175, 129-139.

Tennant, R.W., Ashby, J., 1991. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the US National Toxicology Program. Mutat. Res. 257, 209-227.

Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D., Giammanco, M., 2007. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chem. 104, 466-479.

Trotta, V., Calboli, F.C., Ziosi, M., Guerra, D., Pezzoli, M.C., David, J.R., Cavicchi, S., 2006. Thermal plasticity in Drosophila melanogaster: a comparison of geographic populations. BMC Evol. Biol. 6, 67.

Turner, S.D., Tinwell, H., Piegorsch, W., Schmezer, P., Ashby, J., 2001. The male rat carcinogens limonene and sodium saccharin are not mutagenic to male Big BlueTM rats. Mutagenesis 16, 329-332.

Vallejo, F., Larrosa, M., Escudero, E., Zafrilla, M.P., Cerdá, B., Boza, J., García-Conesa, M.T., Espín, J.C., Tomás-Barberán, F.A., 2010. Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. J. Agr. Food Chem. 58, 6516-6524.

Van der Merwe, J., Joubert, E., Richards, E., Manley, M., Snijman, P., Marnewick, J., Gelderblom, W., 2006. A comparative study on the antimutagenic properties of aqueous extracts of Aspalathus linearis (rooibos), different Cyclopia spp.(honeybush) and Camellia sinensis teas. Mutat. Res. 611, 42-53.

Vecchia, C.L., Bosetti, C., 2006. Diet and cancer risk in Mediterranean countries: open issues. Public Health Nutr. 9, 1077-1082.

Villatoro-Pulido, M., Font, R., De Haro-Bravo, M.I., Romero-Jiménez, M., Anter, J., Bailón, A.D.H., Alonso-Moraga, Á., Del Río-Celestino, M., 2009. Modulation of genotoxicity and cytotoxicity by radish grown in metal-contaminated soils. Mutagenesis 24, 51-57.

Xu, G., Liu, D., Chen, J., Ye, X., Ma, Y., Shi, J., 2008. Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chem. 106, 545-551.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., Adler, P.N., 2008. The multiple-wing-hairs gene encodes a novel GBD–FH3 domain-containing protein that functions both prior to and after wing hair initiation. Genetics 180, 219-228.

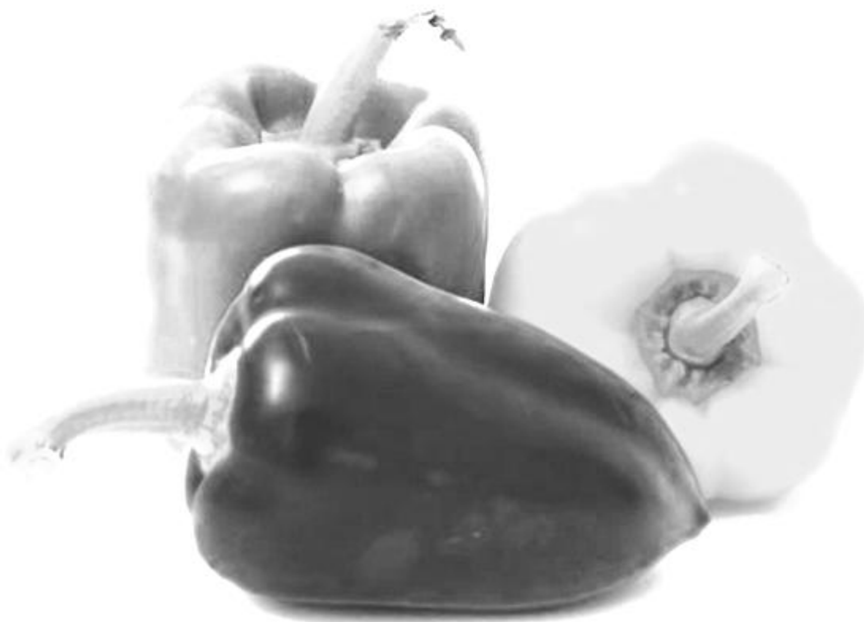
Yang, M., Tanaka, T., Hirose, Y., Deguchi, T., Mori, H., Kawada, Y., 1997. Chemopreventive effects of diosmin and hesperidin on N-butyl-N-(4-hydroxybutyl) nitrosamine-induced urinary-bladder carcinogenesis in male ICR mice. Int. J. Cancer 73, 719-724.

Zordan, M., Graf, U., Singer, D., Beltrame, C., Dalla Valle, L., Osti, M., Costa, R., Levis, A.G., 1991. The genotoxicity of nitrilotriacetic acid (NTA) in a somatic mutation and recombination test in Drosophila melanogaster. Mutat. Res. Lett. 262, 253-261.

CAPÍTULO II: *In vivo* and *in vitro* evaluation for nutraceutical purposes of capsaicin, capsanthin, lutein and four pepper varieties.

Chapter II

Artículo en preparación



ABSTRACT

The purpose of the present research is to give a nutraceutical focus to the use of both worldwide consumed sweet and hot peppers and to contribute with a new corpus of data to the knowledge on the beneficial or prejudicial effects of some molecules contained in this food such as capsaicin, capsanthin and lutein.

Two *in vitro* and *in vivo* models covered several biological targets. The *Drosophila melanogaster* animal model has been used to ascertain (i) the safety by measuring the lack of genotoxicity, (ii) the ability to protect somatic cells from oxidative genetic damage induced hydrogen peroxide (antigenotoxic activity), and (iii) the role on the lifespan extension as an index of integral healthy activity. The HL60 human tumoural cell line was employed to evaluate the chemopreventive cytotoxic effects and the possible proapoptotic activity.

Results showed that: i) none of the tested substances were genotoxic except green hot pepper and capsaicin at the highest concentration assayed (5mg/mL and 11.5 µM respectively), ii) all pepper varieties (except green hot pepper at the highest concentration assayed i.e. 5 mg/mL), capsaicin, capsanthin and lutein are antimutagenic when hydrogen peroxide is used as genotoxin, iii) red sweet pepper variety significantly extend the lifespan and healthspan of *D. melanogaster* at the median concentration (1.25 and 2.5 mg/mL) and green hot pepper significantly reduce the lifespan and healthspan at the highest concentrations (1.5, 2.5 and 5 mg/mL), iv) all pepper varieties inhibit the HL60 cell growth with a

dose-response effect and different IC_{50} (green sweet pepper: 0.55 mg/mL; red sweet pepper: 0.6 mg/mL; green hot pepper: 1.5 mg/mL; red hot pepper: 0.3 mg/mL) but capsaicin, capsanthin and lutein weren´t able to fully inhibit the tumour growth, and v) all pepper varieties and capsaicin exerted proapoptotic effect on HL60 cells.

Based on the results of the present study, we conclude that: (i) capsanthin, lutein and sweet peppers are non-toxic, DNA-safe (non-genotoxic) and show an antimutagenic activity against H_2O_2-DNA damage as an added value. (ii) Lutein and sweet peppers significantly extend the lifespan of *Drosophila melanogaster* (iii) Capsanthin, capsaicin and the four pepper varieties (pungent and non-pungent) are able to inhibit the *in vitro* growth of leukaemia cells (HL60) at different IC_{50}. Additionally, our results support the epidemiological data that positively correlate hot pepper consumption and cancer incidence as Green hot peppers and capsaicin induce DNA damage (genotoxic) and decrease the lifespan of *Drosophila melanogaster.* Therefore, all *in vivo* and *in vitro* assays carried out in the present research point out that: (i) sweet peppers could be suggested as nutraceutical food, (ii) hot peppers should be moderately consumed, and (iii) supplementary studies are necessary to clarify the synergic effect of the carotenoids and capsaicinoids in the food matrix of the red hot pepper.

Keywords: Pepper, capsaicin, carotenoids, genotoxicity, cell viability, DNA-fragmentation

INTRODUCTION

Many studies are focused on studying healthy diets (Rodriguez-Casado, 2014). Epidemiological analyses indicate that diet plays a principal role on genetic damage prevention and longevity extension, although a principal component in longevity for genes is suggested in mutant genotypes for lifespan extension in model organisms experiences (Guarente and Kenyon, 2000).

Capsicum genus fruits are food ingredients and additives widely used around the world due to their versatility to be consumed as fresh vegetable either in salads, cooked meals or dehydrated for spices at different ripening states (green, yellow, orange, red and purple). Its consumption has been traditional for hundreds of years in some areas with estimates of about 40 g/day per capita dietary intake (de Mejía et al., 1998). These fruits vary in size, pungency, color and shape and they are highly valued for these characteristics. Peppers include more than 200 varieties, being two of the most representative species: *Capsicum annuum* and *Capsicum frutescens* (Pino et al., 2007). Medicinal uses of *Capsicum sp* varies in function of ethnicity, species and parts of fruit but in general they have been used as anti-fever, anti-hypertensive, in rheumatism treatment, to improve blood circulation, muscle pain, asthma, stomach upsets, anticancer, anti-obesity and therapy for chilblains, neuralgias and pleurisy (Benítez et al., 2010; Lim, 2012; Luo et al., 2011; Pieroni and Quave, 2005; Pieroni et al., 2004; Srinivasan, 2005). It is well known their antioxidant and nutritive properties

due to the content of bioactive phenolic phytochemicals including capsaicinoids and carotenoids (Topuz and Ozdemir, 2007), whose concentration strongly depends on the ripening state of the fruit (Deepa et al., 2007). Studies on the genomic safety effects of pepper fruit as complex mixture yield negative results for genotoxicity and positive for antigenotoxicity, although in each experience different varieties, assays and mutagens have been used (El Hamss et al., 2003; Laohavechvanich et al., 2006; Ramirez-Victoria et al., 2001; Sim and Han, 2007; Tsuchiya et al., 2011).

Capsaicin (trans-8-methyl-N-vanillyn-6-noneamide; $C_{18}H_{27}NO_3$), is the major alkaloid responsible for the mucosal irritant properties of plant species from the genus *Capsicum* (Barceloux, 2009). This alkaloid has been used for different clinical applications: neuropathic pain, postherapeutic neuralgia, musculoskeletal chronic pain, neurogenic bladder hyperreactivity, gastroprotection, post-operative nausea and vomiting, pruritus in renal dialysis patients and post-operative sore throat (Hayman and Kam, 2008). Capsaicin has been tested as genotoxic or not genotoxic compound depending on the animal and cell system assayed (Chanda et al., 2004; Marques et al., 2002; Surh and Lee, 1995). Several studies have demonstrated its antiproliferative effect and apoptotic induction in different cancer cell lines (Gil and Kang, 2008; Kang et al., 2001; Kim et al., 2004; Maity et al., 2010; Yang et al., 2009).

Capsanthin is one of the carotenoids present in red pepper fruit; and it is included within the C_{40} isoprenoids group which have double bounds in the central polyenic chain and distinctive end groups (β, ε, κ 3-hydroxy-5,6-

epoxide) with characteristic positions for each pigment (Topuz and Ozdemir, 2007). It is also one of the key components responsible for the red colour in the pepper fruits during its ripening process (Deli et al., 2001; Ha et al., 2007) being 7949.48 mg/Kg of dry weight the maximum concentration of this carotenoid in peppers (Hornero-Méndez et al., 2002). It is known its anticancer activity and preventive effect against arteriosclerosis (Maoka et al., 2001; Sun et al., 2005).

Lutein ($C_{40}H_{56}O_2$) is a yellow plant pigment and is one of the carotenoid present in green fruits and vegetables (Hornero-Méndez et al., 2000). The concentration of this carotenoid in green pepper is 173 µg per 100 g of fresh fruit (Perry et al., 2009). This pigment is located in the macula lutea of the human eye (Schalch et al., 2007) and prevents age-related macular degeneration (Marse-Perlman et al., 2001) and cataracts (Gale et al., 2001). It has been demonstrated that lutein has beneficial biological properties in some diseases such as stroke, cardiovascular disease and cancer due to its antioxidative, antimutagenic and antiproliferative properties (Holick et al., 2002; Rafi et al., 2015; Trevithick-Sutton et al., 2006; Wang et al., 2013; Wang et al., 2006).

Capsicum species are one of the most widely consumed vegetables around the world. Therefore, the purpose of this study is to determine the nutraceutic potential of *Capsicum sp*. due to its constituents and at the same time, provide data in order to clarify the controversy of results obtained for capsaicin by different authors. A nutraceutical substance should be able to prevent mutations, exert desmutagenic activity, and eliminate the transformed cells once a tumour is initiated. Besides these

chemopreventive properties, the lifespan extension is one of the most desirable effects of an intended nutraceutical. To achieve these objectives, five types of assays were performed: genotoxicity, antigenotoxicity and life span trials using the *Drosophila* model (*in vivo* assays) and cytotoxicity and DNA fragmentation assays using HL60 cell line (*in vitro* assays).

MATERIAL AND METHODS

Sample preparation of fruits and single compounds

Four pepper varieties, two sweet and two hot, were selected for the present study. The first group (sweet peppers) included red *Lamuyo* and green *Italian Capsicum annuum* varieties and the second group (hot peppers) included red *Chili* and green *Cuernocabra* varieties (*Capsicum frutescens* and *Capsicum annuum* respectively). All the pepper fruits were purchased in a local market, thoroughly washed with tap water and rinsed with distilled water. Finally, samples were freeze-dried at –80 ºC for 3 days, pulverized with a mortar pestle and lyophilized. The single compounds capsaicin and lutein were purchased from Fluka (Cat. Numbers 21748 and 95507 respectively) and capsanthin was purchased from Extrasynthèse (Cat. number 0312S), solved in ethanol and filtered before use.

Genotoxicity and Antigenotoxicity assays

Drosophila melanogaster strains

Two *Drosophila* strains were used, each with a hair marker in the chromosome III:

- *mwh/mwh*, carrying the recessive mutation *mwh* (multiple wing hairs) that produces multiple tricomas per cell (Yan et al., 2008).

- *flr³*/TM3, *Bd^S*, where the *flr³* (flare) marker is a homozygous recessive lethal mutation that produces deformed tricomas because it is viable in homozygous somatic cells once larvae start developing (Ren et al., 2007). See Lindsley and Zimm (2012) for more detailed information on the rest of the genetic markers.

Flies are routinely maintained at 25 ºC, in a homemade meal (1000 ml water, 0.5 g NaCl, 100g yeast, 25 g sucrose, 12g agar-agar, 5 ml propionic acid, 3.5 ml of a 0.2% sulphate streptomycin solution) and with three changes per week.

Treatment Procedures

Genotoxicity assays were performed as described by Graf et al. (1984). Virgin females with the genotype *flr³*/TM3, *Bd^S* were mated to *mwh/mwh* males. Optimal designs were set with 300 females and 150 males each. Flies were allowed to mate for 3 days in order to obtain an optimal production of hybrid eggs at the fourth day after mating. Hybrid eggs were collected from the crosses of optimally fertile flies during an 8-h period in flour-enriched soft medium. Emerged larvae of 72 ± 4 hours were washed from the remaining feeding medium using distilled water and transferred to the treatment vials. These vials contained 0.85 g of dry *Drosophila* Instant Medium (formula 4-24, Carolina Biological Supply, Burlington NC, USA) and 4 mL of the respective test solutions. One hundred larvae were embedded into this medium and fed with different concentrations of the test fruits and single compounds. The concentrations of the different compounds were: 0.625 mg/mL and 5 mg/mL for the pepper varieties, 1.3 and 11.5 µM capsaicin, 1 and 8.5 µM capsanthin and 0.04 and 0.33 µM lutein. The concentrations selected of single compounds fell in the range of

the concentrations as described in the fruit (Estrada et al., 1999; Perry et al., 2009; Topuz and Ozdemir, 2007). Distilled water was used as a concurrent negative control and hydrogen peroxide (150 mM) as the oxidative genotoxicant (Romero-Jimenez et al., 2005).

Antigenotoxicity tests were performed in combined treatments as described before (Graf et al., 1998) by mixing the mutagen (hydrogen peroxide) with the lyophilized samples or the single compounds in appropriate concentrations. Larvae were fed until pupation (about 48 hours) at 25 ± 1 °C. After emergence, adult flies were collected and stored in a 70% ethanol solution until mounting.

Wing scoring

For observation of mutant clones, the wings of transheterozygous flies were mounted on slides with Faure´s solution (30 g Arabic gum, 20 mL Glycerol, 50 g chloral hydrate, 50 mL distilled water) and the hair mutations (spots) were analysed and scored under a photonic microscopy at 400x for the occurrence of single and twin spots. Wing hair mutations (spots) were scored among a total of 24,000 monotricoma cells/wing. Balancer wings (*mwh*/TM3, Bd^S) were also mounted in the positive and genotoxic single treatment concentrations. The mutant spots were spliced into three different categories: (1) small single spots, consisting of 1 or 2 *mwh* or flr^3 cells, which correspond to somatic point mutations, chromosome aberration as well as somatic recombination between both wing genetic markers; (2) large spots, consisting of three or more *mwh* or flr^3 cells, which can be produced by the same processes previously mentioned; and (3) twin

spots, consisting of adjacent *mwh* and *flr*^{3} cells, which are exclusively originated from somatic recombination. The total number of spots was evaluated. This classification has been reported biologically meaningful (Graf et al., 1984). In the case of genotoxic results for single treatments, balancer wings (*mwh/Bd*^{S}) were also evaluated in order to quantify the somatic recombinogenic activity (R) of the substance (Zordan et al., 1991).

Data evaluation and statistical analysis

The genotoxicity/antigenotoxicity results were evaluated according to the U-test of Mann, Whitney and Wilcoxon (α=β=0.05, one sided) using the SPSS Version 15.0 software (SPSS Inc. Headquarters, Chicago, IL, USA). The frequencies of each type of clone per wing were compared with its concurrent negative control and the significance was given at the 5% level.

The somatic recombinogenic activity of the substance was calculated using the following formula:

R = (frequency of mwh spots on the balancer wings/frequency mwh spots on the marker wings) x 100

The antimutagenic effect in combined treatments was evaluated for total spots as proposed by Abraham (1994) by means of the inhibition percentage (IP), according to the formula:

IP = (genotoxine alone - sample plus genotoxine) * 100/genotoxine alone.

Lifespan assays

Strains

Animals with the same genetic background as in genotoxicity assays were used for trials in longevity studies in order to compare the degenerative/antidegenerative effects at both levels. Longevity

experiments were carried out at 25ºC and following the procedure of (Fernández-Bedmar et al., 2011) . Briefly, synchronized transheterozygous larvae of 72 ± 12 hours from *flr^3 x mwh* crosses were washed and separated into groups of 100 individuals in vials with a mixture of Instant Medium and 4mL of the different concentrations of the four varieties of pepper (*Italian*, *Lamuyo*, *Cuernocabra* and *Chili*) and the three selected single compounds (capsaicin, capsanthin and lutein). Emerging flies were anesthetized under CO_2, separated in single-sex groups of 10 individuals and transferred to 1mL longevity glass vials. Four replicates were used during lifespan assays for each control and different concentrations of the test peppers and single compounds. The survivals were counted and the medium renewed twice a week.

Data evaluation and statistical analysis

Data were evaluated following the non-parametric Kaplan-Meier test estimate of the survival function for each concentration and concurrent control and were plotted as survival curves. The statistical analyses and significance levels of these curves were assessed with the Log-Rank (Mantel-Cox) method through SPSS 15.0 statistic program (SPSS Inc. Headquarters, Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$.

Viability assays

Cell cultures

The HL60 (human promyelotic leukaemia) cell line was used for the *in vitro* studies of cytotoxicity. Cell line was grown in a humidified incubator (37ºC; 5% CO_2) in complete medium RPMI 1640 (Biowhittaker, BE12-167F), containing 10% heat-inactivated bovine serum (Biowhittaker, de14-801F),

L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (10,000 units of penicillin, 10mg of streptomycin and 25 µg/mL of amphotericin B) from Sigma (A5955). The cultures were plated at a 2.5x 10^5 cells/mL density and passed every 2-3 days.

Assessment of cytotoxic effect

HL60 cells were placed in 12 well culture plates ($1x10^5$ cells/mL) and treated for 72 hours with different concentrations of the different test compounds (2, 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL for *Italian*, *Lamuyo*, *Chili* and *Cuernocabra* peppers, 98, 65, 49, 12 and 3 µM for capsaicin, 7, 3.5, 1.75, 0.87, 0.1 µM for capsanthin and 0.12, 0.06, 0.03, 0.015, 0.008 and 0.004 µM for lutein). Cell growth and viability were assessed following the Trypan blue exclusion method. For monitoring these parameters, 10 µL of treated HL60 cell suspension from each well were mixed with the same volume of Trypan blue solution (Fluka, 93595). Ten microlitres of the mix solution was placed in both chambers of a haemocytometer and the number of living cells was counted under an inverted microscope (Motic, AE30/31) at 100x magnifications. Each experiment was repeated in triplicate, growth curves were established and IC_{50} values were estimated and plotted as survival percentage with respect to the control growing at 72 h.

Assessment of proapoptotic activity

HL60 cells ($1.5x10^6$ cells/mL) were placed in 12-well culture plates and treated with different concentrations of the test compounds for 5 hours. Treated cells were centrifuged at 4000 rpm for 5 minutes and washed with PBS (SIGMA, D8537). DNA was extracted using a commercial DNA extracted

kit (Dominion mbl, 243) and treated with 10 mg/mL RNase at 37ºC for 30 minutes. DNA fragments (1500 ng) were then separated in 2% agarose gel electrophoresis (50 V for 2 h) and stained with ethidium bromide. Finally the internucleosomal fragments were visualized under UV.

RESULTS

Genotoxicity and antigenotoxicity tests

The *Drosophila* wing-spot test was used to assess the safe use of four varieties of pepper fruits and some of their distinctive compounds. All assays of geno/antigenotoxicity of pepper have been carried out using the SMART model as the *Drosophila melanogaster* larvae are able to metabolize a wide range of molecules and complex mixtures (Graf et al., 1984). **Table 1** shows the genotoxicity results obtained for the seven tested substances**.** The total spots per wing frequency of the negative control was 0.162, being in accordance with the range in other studies (Romero-Jimenez et al., 2005). None of the tested substances were genotoxic at the assayed concentrations (0.625 and 5 mg/mL for green sweet pepper, red sweet pepper and red hot pepper varieties, 0.625 mg/mL for green hot pepper, 1.3 µM for Capsaicin, 1and 8.5 µM for Capsanthin, 0.04 and 0.33 for Lutein), except the highest concentration assayed of Cuernocabra variety (5 mg/mL) that reached 0.450 total spots/wing and capsaicin at 11.5 µM that exhibited 0.400 total spots/wing. In order to evaluate the recombinogenic potency of these genotoxic concentrations, the spots per wing were scored in balancer-heterozygous wings. Values of recombinogenicity with respect to the total induced clones for green hot

Table 1. Genotoxicity of four pepper varieties, capsaicin, capsanthin and lutein in the *Drosophila* wing spot test.

Compounds	N	Small spots (1-2 cells)	Large spots (> 2 cells)	Twin spots	Total spots
Negative control (H_2O)	80	0.12 (10)[a]	0.04 (3)	0 (0)	0.16 (13)
Green Sweet Pepper					
0.625 mg/mL	40	0.2 (8)	0.07 (3)	0 (0)	0.27 (11)ns
5 mg/mL	38	0.05 (2)	0 (0)	0 (0)	0.05 (2)ns
Red Sweet Pepper					
0.625 mg/mL	40	0.22 (9)	0 (0)	0.02 (1)	0.25 (10)ns
5 mg/mL	40	0.22 (9)	0.05 (2)	0 (0)	0.27 (11)ns
Green Hot Pepper					
0.625 mg/mL	36	0.25 (9)	0.08 (3)	0 (0)	0.33 (12)ns
5 mg/mL	40	0.37 (15)	0.07 (3)	0 (0)	0.45(18)**
5 mg/mL *Ser*	40	0.17 (7)	0.02 (1)	0 (0)	0.20 (8)
Red Hot Pepper					
0.625 mg/mL	38	0.10 (4)	0.03 (1)	0.03 (1)	0.16 (6)ns
5 mg/mL	36	0.17 (6)	0 (0)	0.03 (1)	0.20 (7)ns
Capsaicin					
1.3 µM	40	0.22 (9)	0 (0)	0 (0)	0.22 (9)ns
11.5 µM	40	0.40 (16)	0 (0)	0 (0)	0.40 (16)**
11.5 µM *Ser*	40	0.20 (8)	0	0	
Capsanthin					
1 µM	40	0.17 (7)	0.02 (1)	0.02 (1)	0.22 (9)ns
8.5 µM	40	0.30 (12)	0.02 (1)	0 (0)	0.32 (13)ns
Lutein					
0.04 µM	40	0.17 (7)	0.05 (2)	0 (0)	0.22 (9)ns
0.33 µM	40	0.27 (11)	0.05 (2)	0 (0)	0.32 (13)ns

Ser: balancer-heterozygous Beaded Serrate genotype wings; a: number of spots per wing, N: number of scored wings ns, non-significant ($p > 0.05$), *: significant ($p \leq 0$ .05), **: highly significant ($p \leq 0.01$). The data were evaluated by the nonparametric U-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995).

Table 2. Antigenotoxicity of four pepper varieties, capsaicin, capsanthin and lutein in the *Drosophila* wing spot test.

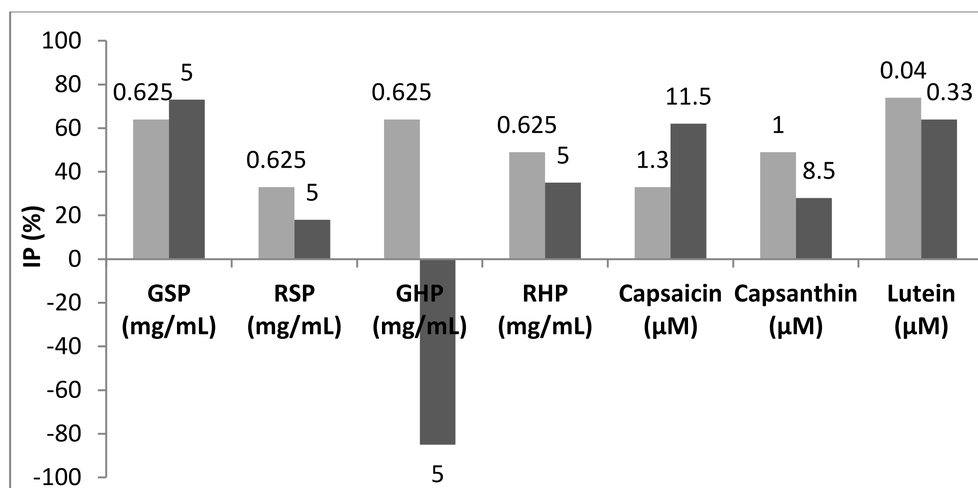
Compounds	N	Small spots (1-2 cells)	Large spots (> 2 cells)	Twin spots	Total spots
Controls					
Negative (H_2O)	80	0.12 (10)	0.04 (3)	0 (0)	0.16 (13)
Positive (H_2O_2, 15 M)	80	0.31 (25)	0.16 (13)	0.01 (1)	0.49 (39)*
Green Sweet Pepper					
0.625 mg/mL	40	0.15 (6)	0.02 (1)	0 (0)	0.17 (7)ns
5 mg/mL	38	0.08 (3)	0.05 (2)	0 (0)	0.13 (5)ns
Red Sweet Pepper					
0.625 mg/mL	40	0.27 (11)	0.05 (2)	0 (0)	0.32 (13)ns
5 mg/mL	40	0.35 (14)	0 (0)	0.05 (2)	0.40 (16)ns
Green Hot Pepper					
0.625 mg/mL	40	0.12 (5)	0.05 (2)	0 (0)	0.17 (7)ns
5 mg/mL	40	0.82 (33)	0.05 (2)	0.02 (1)	0.90 (36)**
Red Hot Pepper					
0.625 mg/mL	40	0.20 (8)	0.05 (2)	0 (0)	0.25 (10)ns
5 mg/mL	38	0.32 (12)	0 (0)	0 (0)	0.32 (12)ns
Capsaicin					
1.3 µM	40	0.30 (12)	0.02 (1)	0 (0)	0.32 (13)ns
11.5 µM	38	0.16 (6)	0.03 (1)	0 (0)	0.19 (7)ns
Capsanthin					
1 µM	40	0.20 (8)	0.05 (2)	0 (0)	0.25 (10)ns
8.5 µM	40	0.30 (12)	0.05 (2)	0 (0)	0.35 (14)*
Lutein					
0.04 µM	32	0.09 (3)	0.03 (1)	0 (0)	0.12 (4)ns
0.33 µM	40	0.12 (5)	0.02 (1)	0 (0)	0.17 (7)ns

The data were evaluated by the non-parametric U-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995). ns: non-significant ($P > 0.05$); *: significant ($P \leq 0.05$); **: highly significant ($P < 0.01$)

pepper and capsaicin were 56% and 50% respectively. **Table 2** shows the antimutagenic effects of four pepper varieties, capsaicin, capsanthin and lutein against hydrogen peroxide. Hydrogen peroxide was used as positive control because it is a well-known mutagen (Driessens et al., 2009). The mutation rate of this genotoxine was 0.487 spots/wing, which is in agreement with other authors that used the same genetic background

(Anter et al., 2011; Tasset-Cuevas et al., 2013). All pepper varieties, except green hot pepper at the highest concentration, were able to inhibit the genotoxic effect of hydrogen peroxide (**Figure 1**). The highest inhibition percentages were for green sweet pepper (64% and 73%), where a dose-effect was observed for this variety. Green hot pepper showed an inhibition percentage of 64 and -85%. The inhibition percentages of red hot pepper (49 and 35%) were higher than the inhibition percentages of red sweet pepper (33% and 18%). Capsaicin inhibited the oxidative damage induced by hydrogen peroxide, a dose-effect was observed for this substance and its inhibition percentages were 33 and 62% at 1.3 and 11.5 µM respectively. Capsanthin showed a high antigenotoxic effect at the lowest concentration (1 µM) with an inhibition percentage of 49%. The inhibition percentages for lutein at 0.04 and 0.33 µM were as high as 74% and 63% respectively (**Figure 1**).

Figure 1. **Inhibition percentages of four pepper varieties (GSP: green sweet pepper, RSP: red sweet pepper, GHP: green hot pepper and RHP: red hot pepper) and their distinctive compounds against H_2O_2-induced genetic damage**.



Longevity assays

Drosophila melanogaster is one of the commonly used models to investigate the genetic determinants of aging (Minois, 2006). The dose-effect relationships of different tested samples are plotted as survival curves and shown in **Figure 2 and Figure 3**. The hot pepper varieties had negative effect on lifespan at the highest assayed concentrations, green sweet pepper induced lifespan maintenance and red sweet pepper had positive effect on lifespan. As shown in **Table 3**, hot varieties reduced the mean lifespan but only supplementation with 1.25, 2.5 and 5 mg green hot pepper/mL diet were significantly different from the negative control, decreasing the mean lifespan by 26%, 21% and 16% respectively. Conversely, the mean lifespan is extended by 15% and 19% when flies are fed with red sweet pepper at the mean concentrations (1.25 and 2.5 mg/mL respectively).

Capsaicin and capsanthin decreased the mean lifespan but only supplementation with 4.3 µM capsanthin diet, significantly reduced the mean lifespan by 12%. Lutein extends the mean lifespan at all concentrations except at 0.16 µM, but these results were no significant (**Table 4**).

Figure 2 Lifespan curves of *Drosophila melanogaster* fed with different concentrations of sweet and hot pepper varieties over time.

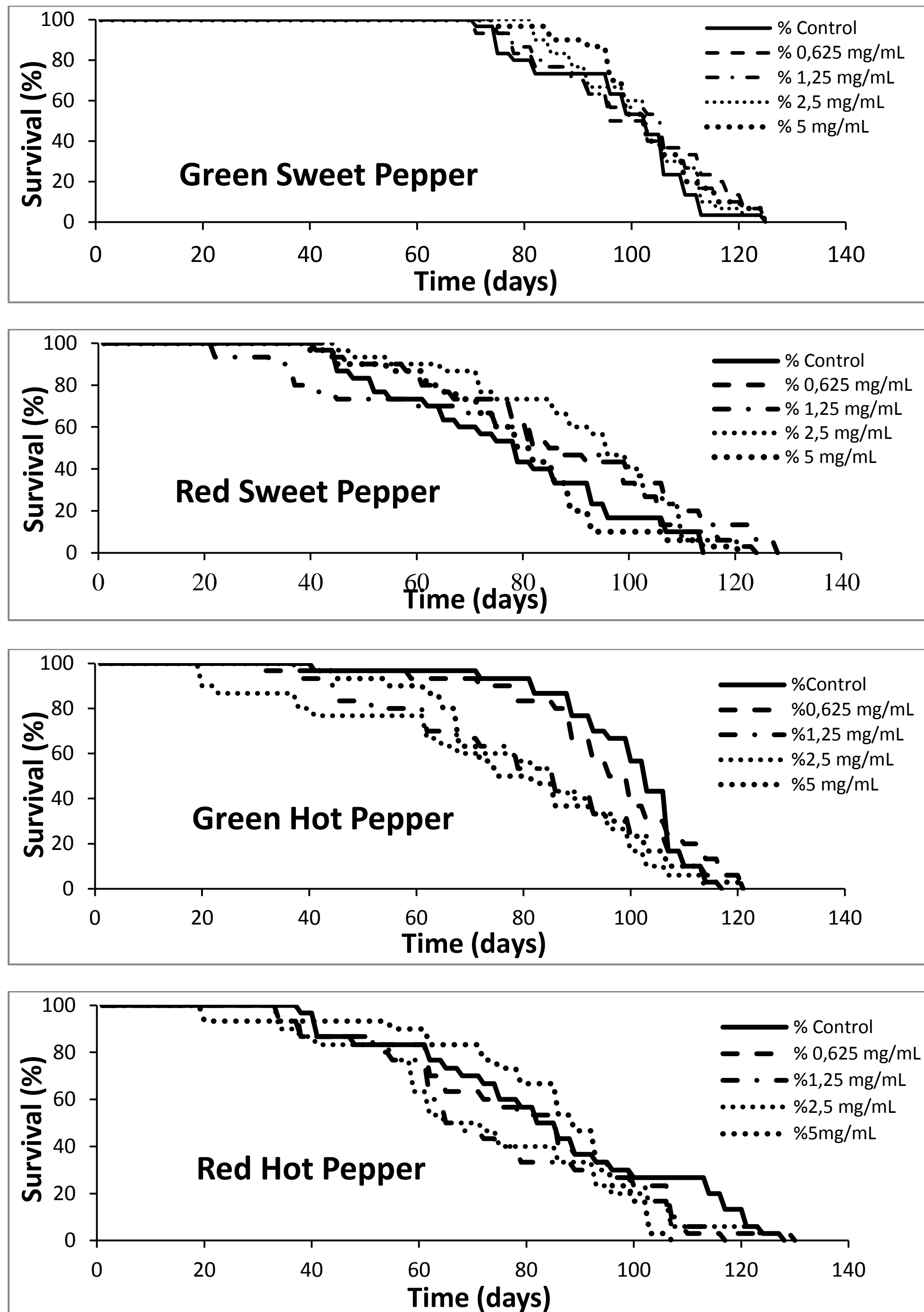
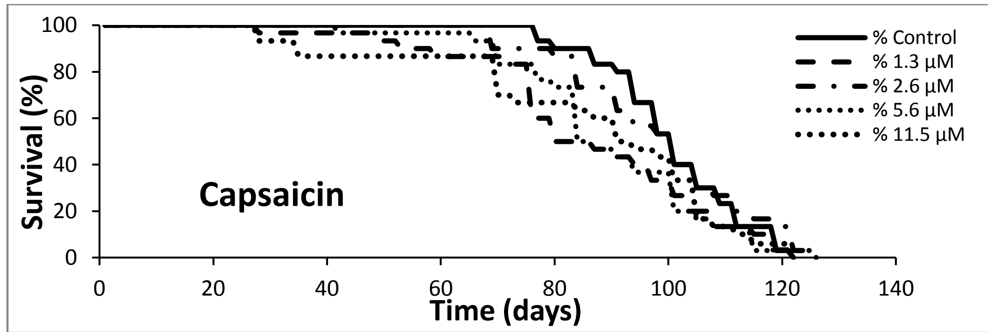
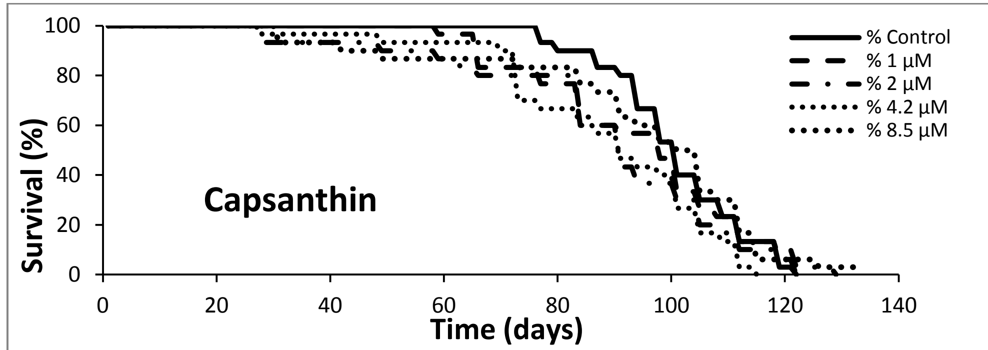


Figure 3 Lifespan curves of *Drosophila melanogaster* fed with different concentrations of capsaicin, capsanthin and lutein over time.





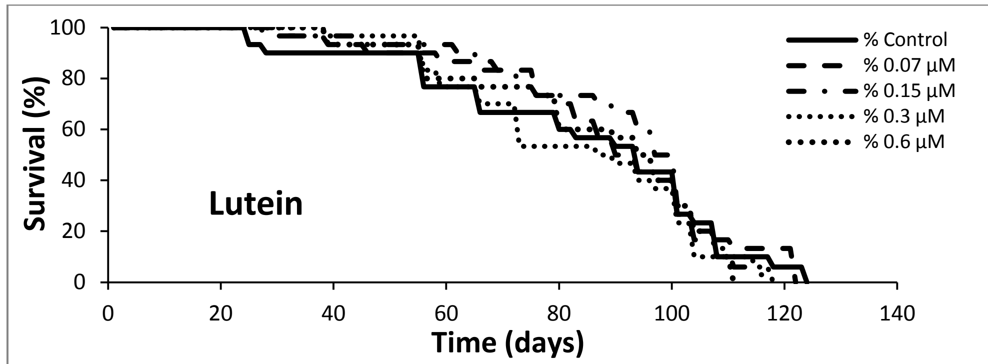


Table 3. Effect of different pepper varieties on the survival time of *Drosophila melanogaster*.

	Mean Lifespan (days)	Mean lifespan difference (%)[a]	Healthspan (75th percentile) (days)	Healthspan difference (%)[a]
RHP(mg/mL)				
Control	84.2 ± 5.0		71.4 ± 4.1	
0.625	80.9 ± 5.0	-4	70.0 ± 4.6	-2
1.25	77.5 ± 4.4	-8	65.2 ± 3.2	-9
2.5	74.0 ± 5.0	-12	61.7 ± 3.9	-13
5	83.2 ± 4.0	-1	76.4 ± 4.6	7
GHP (mg/mL)				
Control	98.6 ± 2.8		94.2 ± 3.3	
0.625	94.6 ± 3.5	-4	87.5 ± 3.7*	-7
1.25	73.2 ± 3.2***	-26	68.3 ± 4.0***	-27
2.5	78.1 ± 5.3**	-21	67.5 ± 6.0***	-28
5	82.4 ± 3.9*	-16	72.5 ± 3.2***	-23
GSP (mg/mL)				
Control	98.8 ± 2.5		93.6 ± 2.7	
0.625	99.9 ± 3.0	1	92.7 ± 2.7	-1
1.25	100.8 ± 2.8	2	94.4 ± 3.0	1
2.5	101.5 ± 2.2	3	96.2 ± 2.0	3
5	103.4 ± 2.1	5	98.2 ± 1.8	5
RSP (mg/mL)				
Control	76.9 ± 4.2		66.7 ± 3.6	
0.625	88.6 ± 4.1	15	78.4 ± 4.1*	18
1.25	88.5 ± 5.7*	15	75.4 ± 5.8*	13
2.5	91.6 ± 3.7*	19	83.9 ± 3.9***	26
5	79.1 ± 3.4	3	71.5 ± 3.1	7

[a] The difference was calculated by comparing treated flies to the concurrent water control. Positive numbers indicate lifespan increase and negative numbers indicate lifespan decrease. *p≤0.05, **p≤0.01, ***p≤0.001 significances obtained by using of the log rank (Mantel-Cox) tests.

Table 4. Effects of capsaicin, capsanthin and lutein on the survival time of *Drosophila melanogaster*.

	Mean lifespan (days)	Mean lifespan difference (%)[a]	Healthspan (75th percentile) (days)	Healthspan difference (%)[a]
Capsaicin (μM)				
Control	100.6 ± 2.2		95.2 ± 1.9	
1.3	92.4 ± 4.1	-8	82.7 ± 4.0**	-13
2.7	97.1 ± 3.8	-3	89.2 ± 4.0	-6
5.7	89.8 ± 3.2	-11	82.2 ± 2.9**	-14
11.5	86.4 ± 4.9	-14	77.2 ± 5.4*	-19
Capsanthin (μM)				
Control	100.6 ± 2.2		95.2 ± 1.9	
1	94.4 ±3.4	-6	86.1 ± 3.1	-9
2	89.4 ± 4.5	-11	80.0 ± 4.7**	-16
4.3	88.7 ± 3.6*	-12	81.1 ± 3.8**	-15
8.5	94.3 ± 4.7	-6	85.9 ± 5.3	-10
Lutein (μM)				
Control	86.1 ± 4.9		76.0 ± 5.1	
0.04	88.6 ± 4.1	3	80.1 ± 4.3	5
0.08	91.1± 3.8	6	84.4 ± 4.4	11
0.16	83.7 ± 4.0	-3	74.7 ± 3.9	-2
0.33	86.8 ± 4.0	1	79.1 ± 4.4	4

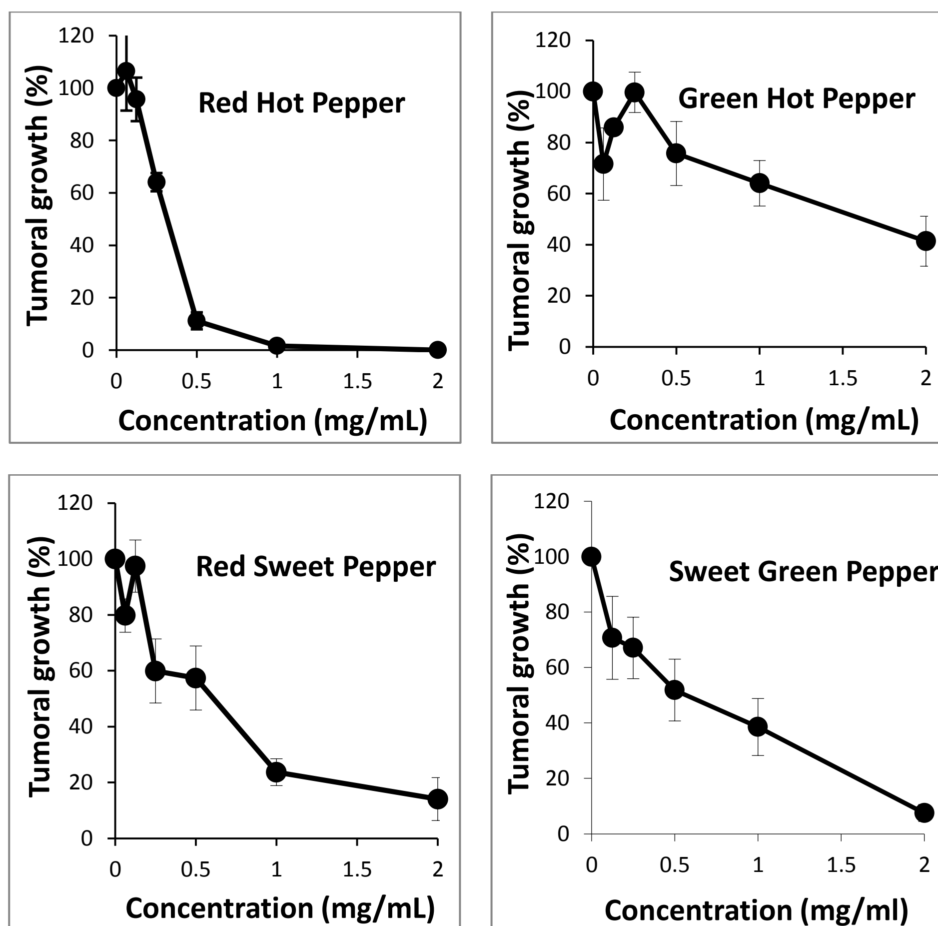
[a] The difference was calculated from comparing treated flies with the respective water control. Positive numbers indicate lifespan increase and negative numbers indicate lifespan decrease. *p<.05, **p<.01, ***p<.001 significances obtained by using of the long-rank (Mantel-Cox) test.

In order to evaluate the healthspan (part of the graphic where a displacement to the right of the survival curves top can be observed and characterized by low and more or less constant age-specific mortality rate value (Soh et al., 2007)), the 75th percentile was analyzed. All concentrations (0.625, 1.25, 2.5 and 5 mg/mL) of green hot pepper significantly decreased healthspan by 7%, 27%, 28% and 23% respectively; nevertheless, red sweet pepper significantly extend healthspan by 18%, 13% and 26% at the lowest and mean concentrations (**Table 3**). Capsaicin significantly decreased healthspan at 1.3, 5.7 and 11.5 µM by 13%, 14% and 19% respectively. Capsanthin also significantly decreased healthspan at mean concentrations (2 and 4.3 µM) by 16% and 15 % respectively. Lutein extends lifespan at the lowest concentrations and at the highest concentration by 5%, 11% and 4% respectively but these results were non-significant (**Table 4**).

Assessment of the cytotoxic effects and apoptosis

The ability of different pepper varieties and their distinctive compounds to inhibit the growth of HL60 cells was also studied. HL60 cells were incubated with different concentrations of the tested substances for 72 h. As shown in **Figure 4**, all pepper varieties inhibited the HL60 cell growth with a dose-response effect; however the IC_{50} values were different between the varieties. The IC_{50} of Cuernocabra variety (green hot pepper) was the highest value (1.5 mg/mL) and Chili variety (red hot pepper) reached the

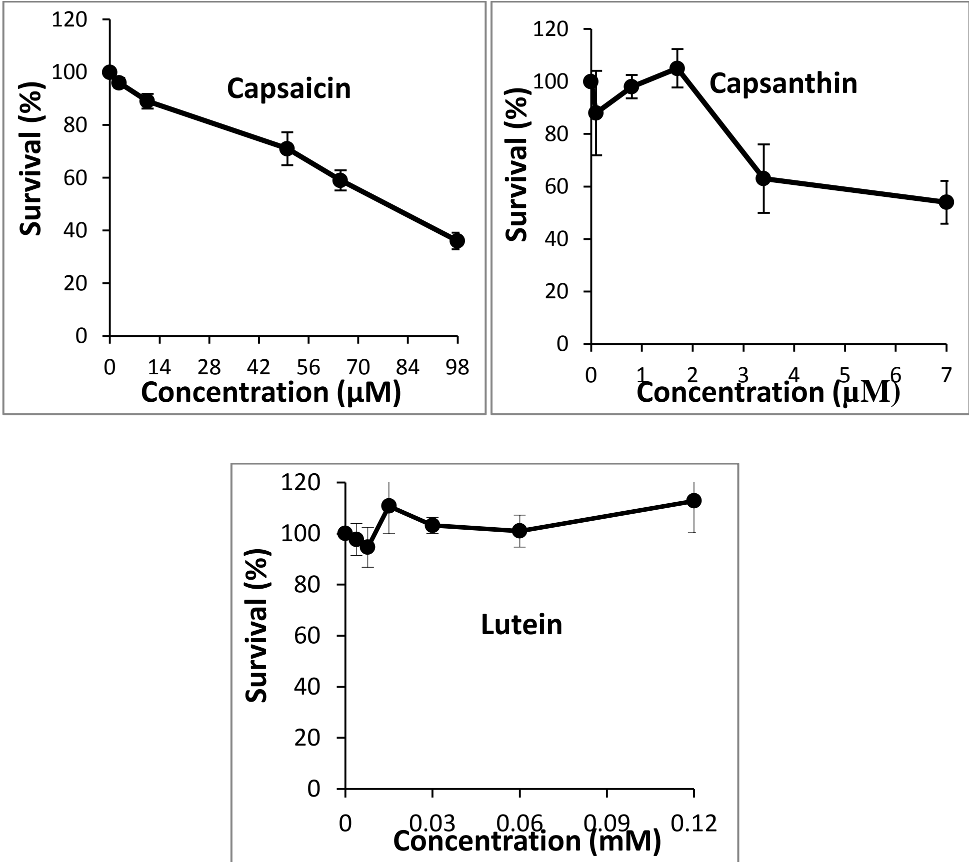
Figure 4. **Cytotoxicity of four varieties of pepper fruits on HL60 cells.**



lowest IC_{50} value (0.3 mg/mL). Italian and Lamuyo varieties (green and red sweet pepper respectively), presented similar IC_{50} (0.55 and 0.6 mg/mL respectively). Among the four tested varieties, Chili was the most effective to inhibit the tumour growth (100% at the highest concentration). **Figure 5**

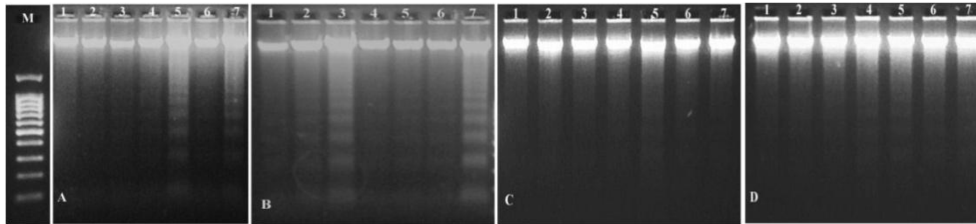
shows the effects of capsaicin, capsanthin and lutein on cell viability. These compounds were not able to fully inhibit the tumour growth. Capsaicin exhibited a dose-response effect and its IC_{50} was 78 µM. Capsanthin didn´t present a clear dose-response effect. The IC_{50} of this compound was reached only at the highest tested concentration (7 µM). Lutein didn´t show a cytotoxic effect in HL60 cell model.

Figure 5. Cytotoxicity of capsaicin, capsanthin and lutein on HL60 cells.



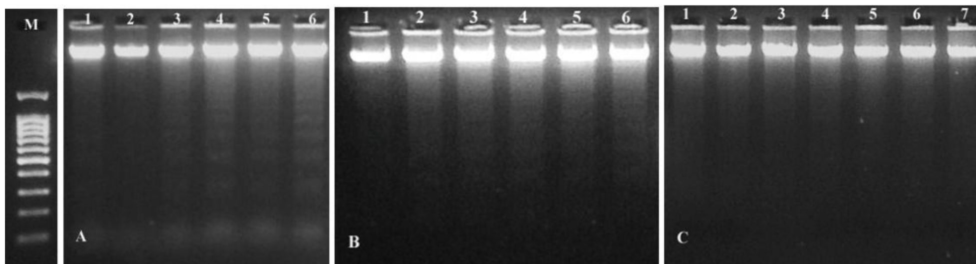
The possible proapoptotic activity of different pepper varieties, capsaicin, capsanthin and lutein has been assayed through DNA fragmentation test. This test can be used to differentiate the apoptosis and necrosis processes and it is characterized by formation of internucleosomal 180-200 bp units. **Figure 6** shows the electrophoresis of the genomic integrity in HL60 cells treated for 5 h with different concentrations of different pepper varieties. DNA fragmentation was found in the HL60 cells treated with red and green hot pepper (0.0625-2 mg/mL) and a weak DNA fragmentation was observed at the three highest concentrations of red sweet pepper (0.5, 1 and 2 mg/mL) and in all concentrations of green sweet pepper (0.25, 0.5, 1 and 2 mg/mL) with the exception of the lower concentration. Internucleosomal fragments were observed in HL60 cells treated with capsaicin at all concentration with the exception of the lower one. Treatment with capsanthin and lutein did not result in the typical DNA laddering in HL60 cells (**Figure 7**).

Figure 6. DNA fragmentation induced in HL-60 cells by four varieties of pepper fruits. HL60 human leukemia cells were exposed for 5 hours to different concentrations of test peppers. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min.



Red hot pepper (A): Control (lane 1), 0.0625 mg/mL (lane 2), 0.125 mg/mL (lane 3), 0.25 mg/mL (lane 4), 0.5 mg/mL (lane 5), 1 mg/mL (lane 6), 2 mg/mL (lane 7). Green hot pepper (B): Control (lane 1), 0.0625 mg/mL (lane 2), 0.125 mg/mL (lane 3), 0.25 mg/mL (lane 4), 0.5 mg/mL (lane 5), 1 mg/mL (lane 6), 2 mg/mL (lane 7). Red sweet pepper (C): Control (lane 1), 0.0625 mg/mL (lane 2), 0.125 mg/mL (lane 3), 0.25 mg/mL (lane 4), 0.5 mg/mL (lane 5), 1 mg/mL (lane 6), 2 mg/mL (lane 7). Green sweet pepper (D): Control (lane 1), 0.0625 mg/mL (lane 2), 0.125 mg/mL (lane 3), 0.25 mg/mL (lane 4), 0.5 mg/mL (lane 5), 1 mg/mL (lane 6), 2 mg/mL (lane 7).

Figure 7. DNA fragmentation induced in HL-60 cells by capsaicin, capsanthin and lutein. HL60 human leukemia cells were exposed for 5 hours to different concentrations of test compounds. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min.



Capsaicin (A): Control (lane 1), 3µM (lane 2), 12 µM (lane 3), 49 µM (lane 4), 65 µM (lane 5), 98 µM (lane 6). Capsanthin (B): Control (lane 1), 0.1 µM (lane 2), 0.85 µM (lane 3), 1.7 µM (lane 4), 3.4 µM (lane 5), 6.8 µM (lane 6). Lutein (C): Control (lane 1), 0.03 µM (lane 2), 0.007 µM (lane 3), 0.014 µM (lane 4), 0.029 µM (lane 5), 0.061 µM (lane 6), 0.12 µM (lane 7).

DISCUSSION

Healthy diets have been a research focus by many authors and have been directly related to the prevention of chronic diseases such as heart diseases and cancer and to the lifespan extension as well (Olsen et al., 2011; Pérez-López et al., 2009; Scarborough et al., 2010). Peppers have been introduced widely around the world from Mexico in the 16th century and included in the traditional American, Asian, European and African diets (Basu et al., 2003).

The first step to consider a substance as nutraceutical is to confirm its role on DNA safety in order to be consumed. The DNA damage caused by oxidative agents accounts for an important portion of harmful mutagenic events. Hydrogen peroxide is a typical DNA damage-inducing agent (Benhusein et al., 2010). This genotoxine induces mitotic recombination in lymphocytes (Turner et al., 2003), somatic mutation, recombination and microsatellite instability germ cells in *Drosophila melanogaster (López et al., 2002; Romero-Jimenez et al., 2005)* and affects to cytoskeletal proteins (Courgeon et al., 1993). Our results confirm that this oxidant agent exhibits mutagenic and recombinogenic activity in somatic cells of *Drosophila sp*. being this organism an excellent *in vivo* genetic model system to be used in genotoxicity and antigenotoxicity studies. Peppers have a high phenolic content among other antioxidant components (Topuz and Ozdemir, 2007) and have been shown to exert free radical-scavenging activity although at different rates depending on the variety and sensorial classification (pungent or non-pungent) (Chuah et al., 2008; de Jesús Ornelas-Paz et al., 2013; Sun et al., 2007; Zhuang et al., 2012). Few genotoxicity studies have

been carried out using pepper fruit as a complex mixture. These studies yielded negative results for different varieties, for both hot and sweet peppers (Poblano, red chili spur, bird chili, green bell and sweet pepper varieties). Our results are in agreement with these except for the positive value found in the green hot pepper at the highest concentration in our experiments. Although the SMART test has been used in all these researches, this apparent discrepancy can be due to the different flies genetic backgrounds used, the concentrations chosen and the part of the fruit selected in each case. Ramirez-Victoria et al. (2001) obtained negative results for Poblano variety (green hot pepper) juice using standard and high bioactivation *Drosophila melanogaster* crosses. In addition, other study with three varieties of *Capsicum annuum* (red chili spur pepper, green bell pepper and sweet pepper) and one variety of *Capsicum frutescens* (bird chili pepper) yielded negative genotoxic results using both standard and high bioactivation crosses although the authors did not show data (Laohavechvanich et al., 2006). El Hamss et al. (2003) also obtained negative results for genotoxicity of bell pepper using standard and high bioactivation *Drosophila melanogaster* crosses. Nevertheless, Tsuchiya et al. (2011), reported that red chili pepper was mutagenic by the Ames test in TA98, TA100, TA1537 and TA1535 *Salmonella* strains, although mutagenic activity levels were low.

The lack of genotoxic activity for most peppers and concentrations confirms their safe use. The following step in the searching of a real nutraceutical substance could be to demonstrate that such a natural product is able to protect against the oxidative damage. Sim and Han

(2007), showed antioxidant activities of red pepper pericarp and red pepper seeds extracts using different antioxidant *in vitro* assays. Besides the genotoxicity assays, combined treatments with hydrogen peroxide have been designed in order to test the antioxidant-antigenotoxic potential of the selected peppers. The results are in agreement with those provided by other authors: (i) Ramirez-Victoria et al. (2001), demonstrated that green Poblano pepper variety inhibited DNA damage induced by methyl urea and sodium nitrite in *Drosophila sp*. ; (ii) Laohavechvanich et al. (2006), also obtained positive results for antigenotoxicity against the carcinogen urethane but the antigenotoxicity levels were lower than in the former research and (iii) bell peppers reduced the mutational events induced by methylmetanesulfonate and ethyl carbamate (El Hamss et al., 2003). Our results are in agreement with these other authors and indicate that peppers have antigenotoxic activity with the exception of the green hot pepper at the highest concentration. This unique difference with the former authors could be due to the different genotoxine and the genetic background used in the different researches. In addition, the genotoxicity and lack of antigenotoxicity in this green hot pepper variety suggest that capsaicin content could be the responsible for the DNA damage observed in green hot pepper and capsaicin treatments. In fact, this capsaicinoid was genotoxic at the highest concentration and the green hot pepper as well. Nevertheless, the red hot variety was non-genotoxic in spite of its capsaicin content, suggesting that the red colorants content counteract the genotoxic effect caused by capsaicin.

Topical use of capsaicin has been suggested as an effective pain management adjunct with therapeutic potential for rheumatoid arthritis, osteoarthritis, neuralgias, diabetic neuropathy. This palliative use is due to the initial neuronal excitation evoked by capsaicin followed by a long-lasting refractory period; during which time the previously excited neurons are no longer responsive to a broad range of apparently unrelated stimuli (Szallasi and Appendino, 2004).

Conflicting both epidemiologic and basic research studies suggest that capsaicin might play a role in either preventing or causing cancer, although most of the studies relates capsaicin with cancer. There are evidences that capsaicin may not be safe for humans in long term topical applications (Bode and Dong, 2011). Furthermore, and despite the statistical limitations, all the epidemiological studies have continually associated capsaicin to a cancer promoting or causative effect. López-Carnllo et al. (1994), found that the consumption of red chili powder was a risk factor for cancer of the oral cavity, pharynx, esophagus and larynx in India. Significantly higher rates were observed for stomach and liver cancer in cultural groups of the U.S. who consume high levels of pepper compared with controls who consume low amounts of hot peppers (Archer and Jones, 2002). Our results for both capsaicin and hot pepper in *Drosophila melanogaster* are according to this. Therefore, our assays are able to detect specific genotoxicity and to associate it with the epidemiological data in humans.

The intervention of the colorful carotenoids compounds on decreasing cancer risk has widely been demonstrated (Donaldson, 2004). Antigenotoxicity studies using capsanthin as DNA-protecting agent are

available, although the present research is the first study providing results regarding genotoxicity. Our results are in disagreement with those of Takahashi et al. (2001), who did not detect antigenotoxic effect of this carotenoid on DNA of *Drosophila sp.* against different carcinogens. The difference could be due to the fact that they used other mutagens(2-amino-3-methylimidazo[4,5-f]quinolone, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, 2-acetylaminofluorene, aflatoxin B1, 7,12-dimethylbenzo[a]anthracene, N-nitrosodimethylamine, N-methyl-N-nitrosourea, 4-nitroquinoline N-oxide) and tests (*Drosophila in vivo* DNA repair). Further, our results are in concordance with the epidemiological studies about relationship between red fruit consumption and cancer risk (Giovannucci et al., 2002).

Lutein has been shown to be an excellent antioxidant and non-mutagenic in prokaryotes at 334, 668 and 1335 µg/plate using the standard Ames test in TA97, TA98, TA100 and TA102 strains in the presence or absence of S9 mix and have anti-mutagenic effect on mutagenicity induced by 2-aminofluorene and cyclophosphamide in TA98 and TA100 strains (Wang et al., 2006). Besides this, this molecule is able to inhibit reactive oxygen species (superoxide and hydroxyl radicals) (Trevithick-Sutton et al., 2006) being this characteristic associated to its high number of conjugated double bonds (Conn et al., 1991). Lutein is also safe when it is evaluated in animal models like rats yielding non-toxicologically relevant findings (Ravikrishnan et al., 2011). Nevertheless, Kalariya et al. (2009) showed that lutein has a DNA damage effect in a dose-and time-dependent manner in ARPE-19 cells (human retinal pigment epithelial cells) at a concentration

ranging from 10-50 µM. The results obtained for us in the eukaryote SMART for lutein indicated that it was not genotoxic and was antigenotoxic, therefore, it is safe and protect to the oxidative DNA damage caused by H_2O_2.

Oxidative stress plays an important role on the lifespan of different *Drosophila melanogaster* strains when adding specific chronic diets in controlled environments (Fleming et al., 1992). *Drosophila* was the first genetic system in which enhanced stress resistance was linked to increased lifespan (Service et al., 1985). In this work, we describe for the first time the effects on the lifespan of *Drosophila melanogaster* of dietary supplementation with different pepper varieties (two pungent and two non-pungent), capsaicin and capsanthin. This arthropod can be used as an aging model system because it shares similar metabolic pathways to human (Anh et al., 2011), such as analogous organ systems that control nutrient uptake, storage and metabolism (Baker and Thummel, 2007). Furthermore, *Drosophila* is costless and a rapid-growing organism. In this study, we demonstrate that hot pepper varieties decrease life/health span in *Drosophila melanogaster* in a dependent-dose manner but only green pepper results were significantly different from the negative control. On the other hand, red sweet pepper variety increase life/health span in this organism at the median concentrations. The lifespan results obtained for capsaicin could partially explain the results obtained for hot varieties. Capsanthin reduces lifespan and health span, therefore, the life/health span extension of *Drosophila* by red sweet pepper supplementation could be due to other components present in the red pepper tissues and lacking

in the green pepper matrix. Zhang et al. (2011), showed that lutein significantly extend mean lifespan and healthspan at 0.1 mg/mL. The observed tendency in our results is in agreement with these authors although statistically non-significant. In general, the simple compounds do not decrease significantly the lifespan, therefore, there should be other components influencing on the lifespan of *Drosophila melanogaster*.

The negative effect of green hot pepper and positive effect of red sweet pepper on the longevity trials of *Drosophila sp*. are in agreement with the genotoxicity results for these vegetables. Hence, the *in vivo* assays results are congruent as genotoxic substances significantly reduced the lifespan in *Drosophila melanogaster*.

Additionally, our results show that the samples tested exert an overall cytotoxic effect on HL60 tumour cells. The present study shows the first cytotoxic assays with fresh pepper fruits as a complex mixture. Our results are in agreement with those of Motohashi et al. (2003) who found positive results for cytotoxicity for different tumour cell lines (HSC-2, HSC-3, HSG and HL60) using different red sweet pepper (Anastasia red, *Capsicum annuum* L. var. *angulosum* Mill.) hexane, acetone and methanol extracts. Obtaining very similar IC_{50} in the case of HL60 cell line (0.5 and 0.6 mg/mL for both studies).The simple compounds were not able to fully inhibit the tumour cell growth % at the tested concentrations (according to the concentrations present in the pepper fruit). There are many cytotoxicity studies using different cancer cell lines (HL60, SNU-1, SHSY-5Y, PC-3, LNC_aP, AGS, A172, SK-Hep-1) all of them yielding positive results for capsaicin (Gil and Kang, 2008; Ito et al., 2004; Jung et al., 2001; Kang et al., 2001; Kim et

al., 1997; Lo et al., 2005; Mori et al., 2006; Richeux et al., 1999). Precisely, Kang et al. (2001) using HL60 cells obtained an IC_{50} like ours.

Capsanthin decreases cell viability of leukemia K562 cells, EBV-EA lymphoblastoid cells, PC3 and DU 145 prostate cancer cells (Kotake-Nara et al., 2001; Maoka et al., 2001; Zhang et al., 2011) at different rates. Nevertheless, Murakami et al. (2000) showed that capsanthin has not a notable cytotoxic effect on HL60 at 25µM. Bridging the gap for the physiological differences among cell lines, our results are more in agreement with those obtained in other cell lines than in those obtained in the HL60 cell line experiments carried out by of Murakami et al. (2000), because capsanthin is able to reduce the cell viability up to 50% at the highest concentration (7 µM).

Lutein has a free radical scavenging activity by 44.1% and IC_{50} at 5.28 µg/mL on HeLa cells (Lakshminarayana et al., 2010). This carotenoid extracted from algae species and Marigold flower petals (*Tagetes erecta* L.) has also showed antiproliferative effect on HCT116 and Hep2 cancer cell lines (Ayyadurai et al., 2013; Cha et al., 2008) and induces HL60 cell differentiation in a dose-dependent manner at 0.1, 1 and 10µM (Gross et al., 1997). Nevertheless lutein extracted from spinach at 5 and 10 µM had minimal effect on cell viability of HL60 cells and only 20 µM concentration decreased the viability by 50% (Ganesan et al., 2011). Lu et al. (2005) also showed that lutein had not effect on the cell growth of LNCaP cells and had minimal effect on PC-3 cells at 8 µmol/L. Our negative results for lutein cytotoxicity are in agreement with the negative results yield by Ganesan et

al. (2011) and Lu et al. (2005). Therefore, lutein has not the ability for cancer growth inhibition at low concentration.

Apoptosis or programmed cell death is a major mechanism of cancer suppression. The induction of the apoptotic mechanism or cell cycle arrest can be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetic damage preinitiated or neoplastic cells from the body. The present work shows the first study on the possible apoptotic effect induced by *Capsicum annuum* fruit as a complex mixture. The hot pepper varieties showed a higher pro-apoptotic internucleosomal fragmentation effect than sweet varieties. The difference between pungent or non-pungent varieties could be due to the capsaicin content of hot varieties because capsaicin also induced DNA fragmentation in HL60 cells. The minimal apoptotic effects observed on DNA of HL60 cells for sweet peppers could be due to the capsanthin content in red variety and another compounds present in green pepper (not lutein).

Capsaicin induces DNA fragmentation in different cancer cell lines: hepatocarcinoma cells (SK-Hep-1), gastric adenocarcinoma cells (AGS), colon cancer cells (HT-29), glioblastoma cells (A172), melanoma cells (B16-F10), prostate cancer cells (PC-3), esophagus epidermoid carcinoma cells (CE 81T/VGH), pancreatic cancer cells (BxPC-3 and AsPC-1), leukemic cells (NB-4, UF-1, Kasumi-1, HL-60, K562, KU812, U937). (Ito et al., 2004; Jun et al., 2007; Jung et al., 2001; Kim et al., 2004; Lee et al., 2000; Lo et al., 2005; Pramanik et al., 2011; Sánchez et al., 2007; Tsou et al., 2006; Wu et al., 2006). Several studies has demonstrated that capsaicin-induced apoptosis is associated with an increase of the intracellular reactive oxygen species

production in NB4 leukemic cell lines playing a significant role in phosphorylation of p53 at the Ser-15 residue (Ito et al., 2004). Tsou et al. (2006) proposed that capsaicin induced decreases in CDK1, cyclin E and cyclin A leading G_0/G_1 arrest and increased the production of reactive oxygen species and Ca^{2+} and decreased MMP levels and cytochrome c release, leading to caspase-3 activity that induced apoptosis in HL-60 cells. Therefore, our results are in agreement with those authors and confirm that capsaicin induces DNA fragmentation in cancer cells.

Few studies have focused to study the apoptotic effect on DNA of cancer cell by capsanthin. This red carotenoid induced significantly cell apoptosis in leukemia K562 cells at 50 µM (Zhang et al., 2011) and in human breast cancer cells (MDA-MB23(HTB26)) at 2 µg/mL (MOLNAR et al., 2004). Our results are in disagreement with these authors and this difference could be due to the different concentrations, cell line genetic backgrounds and specific assays conditions used in each case.

Recent research has shown that dietary lutein can inhibit growth in mouse mammary tumours by regulating angiogenesis and apoptosis (Chew et al., 2002). This yellow carotenoid induces apoptosis in prostate cancer cells (PC3) (Rafi et al., 2015). Our results in HL60 cells are in disagreement with these.

The purpose of the present research work has been to give a nutraceutical focus to the use of both worldwide consumed sweet and hot peppers as well as to question their indiscriminate consumption. Based on the results of the present study, we conclude that: (i) sweet peppers, capsanthin and

lutein are non-toxic, DNA-safe (non-genotoxic) and show an antimutagenic activity against H_2O_2-DNA damage as an added value. (ii) Sweet peppers and lutein extend the lifespan of *Drosophila melanogaster* (iii) All pepper varieties (pungent or non-pungent) capsanthin and capsaicin are able to inhibit the *in vitro* growth of leukaemian cells (HL60) at different IC50. Additionally, our results support the epidemiological data that positively correlate hot pepper consumption and cancer incidence as Green hot peppers and capsaicin induce DNA damage (genotoxic) and decrease the lifespan of *Drosophila melanogaster.* Therefore, all *in vivo* and *in vitro* assays carried out in the present research point out that: (i) sweet peppers could be suggested as nutraceutical food, (ii) hot peppers should be moderately consumed, and (iii) supplementary studies are necessary to clarify the synergic effect of the carotenoids and capsaicinoids in the food matrix of the red hot pepper.

REFERENCES

Abraham, S.K., 1994. Antigenotoxicity of coffee in the Drosophila assay for somatic mutation and recombination. Mutagenesis 9, 383-386.

Anh, N.T.T., Nishitani, M., Harada, S., Yamaguchi, M., Kamei, K., 2011. A Drosophila model for the screening of bioavailable NADPH oxidase inhibitors and antioxidants. Mol. Cell Biochem. 352, 91-98.

Anter, J., Romero-Jiménez, M., Fernández-Bedmar, Z., Villatoro-Pulido, M., Analla, M., Alonso-Moraga, Á., Muñoz-Serrano, A., 2011. Antigenotoxicity, cytotoxicity, and apoptosis induction by apigenin, bisabolol, and protocatechuic acid. J. Med. Food 14, 276-283.

Archer, V.E., Jones, D.W., 2002. Capsaicin pepper, cancer and ethnicity. Med. Hypotheses 59, 450-457.

Ayyadurai, N., Valarmathy, N., Kannan, S., Jansirani, D., Alsenaidy, A., 2013. Evaluation of cytotoxic properties of Curcuma longa and Tagetes erecta on cancer cell line (Hep2). Afr. J. Ph. Pharm. 7, 736-739.

Baker, K.D., Thummel, C.S., 2007. Diabetic larvae and obese flies—emerging studies of metabolism in Drosophila. Cell Metab. 6, 257-266.

Barceloux, D.G., 2009. Pepper and capsaicin (Capsicum and Piper species). Disease-a-Month 55, 380-390.

Basu, S.K., De, A.K., De, A., 2003. Capsicum: historical and botanical perspectives. Capsicum: the genus Capsicum, 1-15.

Benhusein, G.M., Mutch, E., Aburawi, S., Williams, F.M., 2010. Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay. Libyan J. Med. 5.

Benítez, G., González-Tejero, M., Molero-Mesa, J., 2010. Pharmaceutical ethnobotany in the western part of Granada province (southern Spain): Ethnopharmacological synthesis. J. Ethnopharmacol. 129, 87-105.

Bode, A.M., Dong, Z., 2011. The two faces of capsaicin. Cancer Res. 71, 2809-2814.

Conn, P.F., Schalch, W., Truscott, T.G., 1991. The singlet oxygen and carotenoid interaction. J. Photoch. Photobio. B. 11, 41-47.

Courgeon, A.-M., Maingourd, M., Maisonhaute, C., Montmory, C., Rollet, E., Tanguay, R.M., Best-Belpomme, M., 1993. Effect of hydrogen peroxide on cytoskeletal proteins of Drosophila cells: comparison with heat shock and other stresses. Exp. Cell Res. 204, 30-37.

Cha, K.H., Koo, S.Y., Lee, D.-U., 2008. Antiproliferative effects of carotenoids extracted from Chlorella ellipsoidea and Chlorella vulgaris on human colon cancer cells. J. Agr. Food Chem. 56, 10521-10526.

Chanda, S., Erexson, G., Riach, C., Innes, D., Stevenson, F., Murli, H., Bley, K., 2004. Genotoxicity studies with pure trans-capsaicin. Mutat. Res. 557, 85-97.

Chew, B.P., Brown, C.M., Park, J.S., Mixter, P.F., 2002. Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis. Anticancer Res. 23, 3333-3339.

Chuah, A.M., Lee, Y.-C., Yamaguchi, T., Takamura, H., Yin, L.-J., Matoba, T., 2008. Effect of cooking on the antioxidant properties of coloured peppers. Food Chem. 111, 20-28.

de Jesús Ornelas-Paz, J., Cira-Chávez, L.A., Gardea-Béjar, A.A., Guevara-Arauza, J.C., Sepúlveda, D.R., Reyes-Hernández, J., Ruiz-Cruz, S., 2013. Effect of heat treatment on the content of some bioactive compounds and free radical-scavenging activity in pungent and non-pungent peppers. Food Res. Int. 50, 519-525.

de Mejía, E.G., Quintanar-Hernández, J.A., Loarca-Piña, G., 1998. Antimutagenic activity of carotenoids in green peppers against some nitroarenes. Mutat. Res. 416, 11-19.

Deepa, N., Kaur, C., George, B., Singh, B., Kapoor, H., 2007. Antioxidant constituents in some sweet pepper (Capsicum annuum L.) genotypes during maturity. LWT- Food Sci. Technol. 40, 121-129.

Deli, J., Molnár, P., Matus, Z., Tóth, G., 2001. Carotenoid composition in the fruits of red paprika (Capsicum annuum var. lycopersiciforme rubrum) during ripening; biosynthesis of carotenoids in red paprika. J. Agr. Food Chem. 49, 1517-1523.

Donaldson, M.S., 2004. Nutrition and cancer: a review of the evidence for an anti-cancer diet. Nutr. J. 3, 19.

Driessens, N., Versteyhe, S., Ghaddhab, C., Burniat, A., De Deken, X., Van Sande, J., Dumont, J.-E., Miot, F., Corvilain, B., 2009. Hydrogen peroxide induces DNA single-and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. Endocr-Relat Cancer 16, 845-856.

El Hamss, R., Idaomar, M., Alonso-Moraga, A., Serrano, A.M., 2003. Antimutagenic properties of bell and black peppers. Food Chem. Toxicol. 41, 41-47.

Estrada, B., Pomar, F., Dıaz, J., Merino, F., Bernal, M., 1999. Pungency level in fruits of the Padrón pepper with different water supply. Sci. Hortic. Amsterdam 81, 385-396.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Fleming, J., Reveillaud, I., Niedzwiecki, A., 1992. Role of oxidative stress in Drosophila aging. Mutat. Res. 275, 267-279.

Gale, C.R., Hall, N.F., Phillips, D.I., Martyn, C.N., 2001. Plasma antioxidant vitamins and carotenoids and age-related cataract. Ophthalmology 108, 1992-1998.

Ganesan, P., Noda, K., Manabe, Y., Ohkubo, T., Tanaka, Y., Maoka, T., Sugawara, T., Hirata, T., 2011. Siphonaxanthin, a marine carotenoid from green algae, effectively induces apoptosis in human leukemia (HL-60) cells. BBA-Gen. Subjects 1810, 497-503.

Gil, Y.-G., Kang, M.-K., 2008. Capsaicin induces apoptosis and terminal differentiation in human glioma A172 cells. Life Sci. 82, 997-1003.

Giovannucci, E., Rimm, E.B., Liu, Y., Stampfer, M.J., Willett, W.C., 2002. A Prospective Study of Tomato Products, Lycopene, and Prostate Cancer Risk. J. Natl. Cancer Inst. 94, 391-398.

Graf, U., Abraham, S.K., Guzmán-Rincón, J., Würgler, F.E., 1998. Antigenotoxicity studies in Drosophila melanogaster. Mutat. Res.- Fund. Mol. M. 402, 203-209.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Gross, M.D., Bishop, T.D., Belcher, J.D., Jacobs Jr, D.R., 1997. Induction of HL-60 cell differentiation by carotenoids.

Guarente, L., Kenyon, C., 2000. Genetic pathways that regulate ageing in model organisms. Nature 408, 255-262.

Ha, S.-H., Kim, J.-B., Park, J.-S., Lee, S.-W., Cho, K.-J., 2007. A comparison of the carotenoid accumulation in Capsicum varieties that show different ripening colours: deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. J. Exp. Bot. 58, 3135-3144.

Hayman, M., Kam, P.C., 2008. Capsaicin: a review of its pharmacology and clinical applications. Curr. Anaesth. Crit. Care 19, 338-343.

Holick, C.N., Michaud, D.S., Stolzenberg-Solomon, R., Mayne, S.T., Pietinen, P., Taylor, P.R., Virtamo, J., Albanes, D., 2002. Dietary carotenoids, serum β-carotene, and retinol and risk of lung cancer in the Alpha-Tocopherol, Beta-Carotene cohort study. Am. J. Epidemiol. 156, 536-547.

Hornero-Méndez, D., Costa-García, J., Mínguez-Mosquera, M.I., 2002. Characterization of carotenoid high-producing Capsicum annuum cultivars selected for paprika production. J. Agr. Food Chem. 50, 5711-5716.

Hornero-Méndez, D., Gómez-Ladrón de Guevara, R., Minguez-Mosquera, M.I., 2000. Carotenoid biosynthesis changes in five red pepper (Capsicum annuum L.) cultivars during ripening. Cultivar selection for breeding. J. Agr. Food Chem. 48, 3857-3864.

Ito, K., Nakazato, T., Yamato, K., Miyakawa, Y., Yamada, T., Hozumi, N., Segawa, K., Ikeda, Y., Kizaki, M., 2004. Induction of Apoptosis in Leukemic Cells by Homovanillic Acid Derivative, Capsaicin, through Oxidative Stress Implication of Phosphorylation of p53 at Ser-15 Residue by Reactive Oxygen Species. Cancer Res. 64, 1071-1078.

Jun, H.-S., Park, T., Lee, C.K., Kang, M.K., Park, M.S., Kang, H.I., Surh, Y.-J., Kim, O.H., 2007. Capsaicin induced apoptosis of B16-F10 melanoma cells through down-regulation of Bcl-2. Food Chem. Toxicol. 45, 708-715.

Jung, M.-Y., Kang, H.-J., Moon, A., 2001. Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 downregulation and caspase-3 activation. Cancer Lett. 165, 139-145.

Kalariya, N.M., Ramana, K.V., Srivastava, S.K., van Kuijk, F.J., 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Curr. Eye Res. 34, 737-747.

Kang, S.N., Chung, S.W., Kim, T.S., 2001. Capsaicin potentiates 1, 25-dihydoxyvitamin D 3-and all-trans retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Eur. J. Pharmacol. 420, 83-90.

Kim, C.-S., Park, W.-H., Park, J.-Y., Kang, J.-H., Kim, M.-O., Kawada, T., Yoo, H., Han, I.-S., Yu, R., 2004. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor γ in HT-29 human colon cancer cells. J. Med. Food 7, 267-273.

Kim, J.-D., Kim, J.-M., Pyo, J.-O., Kim, S.-Y., Kim, B.-S., Yu, R., Han, I.-S., 1997. Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. Cancer Lett. 120, 235-241.

Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., Nagao, A., 2001. Carotenoids affect proliferation of human prostate cancer cells. J. Nutr. 131, 3303-3306.

Lakshminarayana, R., Sathish, U.V., Dharmesh, S.M., Baskaran, V., 2010. Antioxidant and cytotoxic effect of oxidized lutein in human cervical carcinoma cells (HeLa). Food Chem. Toxicol. 48, 1811-1816.

Laohavechvanich, P., Kangsadalampai, K., Tirawanchai, N., Ketterman, A., 2006. Effect of different Thai traditional processing of various hot chili peppers on urethane-induced somatic mutation and recombination in Drosophila melanogaster: Assessment of the role of glutathione transferase activity. Food Chem. Toxicol. 44, 1348-1354.

Lee, Y.S., Nam, D.H., Kim, J.-A., 2000. Induction of apoptosis by capsaicin in A172 human glioblastoma cells. Cancer Lett. 161, 121-130.

Lim, T.K., 2012. Edible medicinal and non-medicinal plants. Springer.

Lindsley, D.L., Zimm, G.G., 2012. The genome of Drosophila melanogaster. Academic Press.

Lo, Y.-C., Yang, Y.-C., Wu, I.-C., Kuo, F.-C., Liu, C.-M., Wang, H.-W., Kuo, C.-H., Wu, J.-Y., Wu, D.-C., 2005. Capsaicin-induced cell death in a human gastric adenocarcinoma cell line. World J. Gastroentero. 11, 6254-6257.

López-Carnllo, L., Avila, M.H., Dubrow, R., 1994. Chili pepper consumption and gastric cancer in Mexico: a case-control study. Am. J. Epidemiol. 139, 263-271.

López, A., Xamena, N., Marcos, R., Velázquez, A., 2002. Germ cells microsatellite instability: The effect of different mutagens in a mismatch repair mutant of Drosophila (spel1). Mutat. Res. 514, 87-94.

Lu, Q.-Y., Arteaga, J.R., Zhang, Q., Huerta, S., Go, V.L.W., Heber, D., 2005. Inhibition of prostate cancer cell growth by an avocado extract: role of lipid-soluble bioactive substances. J. Nutr. Biochem. 16, 23-30.

Luo, X.-J., Peng, J., Li, Y.-J., 2011. Recent advances in the study on capsaicinoids and capsinoids. Eur. J. Pharmacol. 650, 1-7.

Maity, R., Sharma, J., Jana, N.R., 2010. Capsaicin induces apoptosis through ubiquitin–proteasome system dysfunction. J. Cell. Biochem. 109, 933-942.

Maoka, T., Mochida, K., Kozuka, M., Ito, Y., Fujiwara, Y., Hashimoto, K., Enjo, F., Ogata, M., Nobukuni, Y., Tokuda, H., 2001. Cancer chemopreventive activity of carotenoids in the fruits of red paprika Capsicum annuum L. Cancer Lett. 172, 103-109.

Marques, S., Oliveira, N., Chaveca, T., Rueff, J., 2002. Micronuclei and sister chromatid exchanges induced by capsaicin in human lymphocytes. Mutat. Res. 517, 39-46.

Marse-Perlman, J.A., Fisher, A.I., Klein, R., Palta, M., Block, G., Millen, A.E., Wright, J.D., 2001. Lutein and zeaxanthin in the diet and serum and their relation to age-related maculopathy in the third national health and nutrition examination survey. Am. J. Epidemiol. 153, 424-432.

Minois, N., 2006. How should we assess the impact of genetic changes on ageing in model species? Ageing Res. Rev. 5, 52-59.

MOLNAR, J., GYÉMANT, N., MUCSI, I., MOLNAR, A., SZAB, M., KÖRTVÉLYESI, T., VARGA, A., MOLNAR, P., TOTH, G., 2004. Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. In Vivo 18, 237-244.

Mori, A., Lehmann, S., O'Kelly, J., Kumagai, T., Desmond, J.C., Pervan, M., McBride, W.H., Kizaki, M., Koeffler, H.P., 2006. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. Cancer Res. 66, 3222-3229.

Motohashi, N., Wakabayashi, H., Kurihara, T., Takada, Y., Maruyama, S., Sakagami, H., Nakashima, H., Tani, S., Shirataki, Y., Kawase, M., 2003. Cytotoxic and multidrug resistance reversal activity of a vegetable,'Anastasia Red', a variety of sweet pepper. Phytother. Res. 17, 348-352.

Murakami, A., Nakashima, M., Koshiba, T., Maoka, T., Nishino, H., Yano, M., Sumida, T., Kim, O.K., Koshimizu, K., Ohigashi, H., 2000. Modifying effects of carotenoids on superoxide and nitric oxide generation from stimulated leukocytes. Cancer Lett. 149, 115-123.

Olsen, A., Egeberg, R., Halkjær, J., Christensen, J., Overvad, K., Tjønneland, A., 2011. Healthy aspects of the Nordic diet are related to lower total mortality. J. Nutr. 141, 639-644.

Pérez-López, F.R., Chedraui, P., Haya, J., Cuadros, J.L., 2009. Effects of the Mediterranean diet on longevity and age-related morbid conditions. Maturitas 64, 67-79.

Perry, A., Rasmussen, H., Johnson, E.J., 2009. Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products. J. Food Compos. Anal. 22, 9-15.

Pieroni, A., Quave, C.L., 2005. Traditional pharmacopoeias and medicines among Albanians and Italians in southern Italy: a comparison. J. Ethnopharmacol. 101, 258-270.

Pieroni, A., Quave, C.L., Santoro, R.F., 2004. Folk pharmaceutical knowledge in the territory of the Dolomiti Lucane, inland southern Italy. J. Ethnopharmacol. 95, 373-384.

Pino, J., González, M., Ceballos, L., Centurión-Yah, A.R., Trujillo-Aguirre, J., Latournerie-Moreno, L., Sauri-Duch, E., 2007. Characterization of total capsaicinoids, colour and volatile compounds of Habanero chilli pepper (Capsicum chinense Jack.) cultivars grown in Yucatan. Food Chem. 104, 1682-1686.

Pramanik, K.C., Boreddy, S.R., Srivastava, S.K., 2011. Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells. Plos One 6, e20151.

Rafi, M.M., Kanakasabai, S., Gokarn, S.V., Krueger, E.G., Bright, J.J., 2015. Dietary Lutein Modulates Growth and Survival Genes in Prostate Cancer Cells. J. Med. Food 18, 173-181.

Ramirez-Victoria, P., Guzman-Rincon, J., Espinosa-Aguirre, J., Murillo-Romero, S., 2001. Antimutagenic effect of one variety of green pepper (Capsicum spp.) and its possible interference with the nitrosation process. Mutat. Res. 496, 39-45.

Ravikrishnan, R., Rusia, S., Ilamurugan, G., Salunkhe, U., Deshpande, J., Shankaranarayanan, J., Shankaranarayana, M., Soni, M.G., 2011. Safety assessment of lutein and zeaxanthin (Lutemax™ 2020): Subchronic toxicity and mutagenicity studies. Food Chem. Toxicol. 49, 2841-2848.

Ren, N., Charlton, J., Adler, P.N., 2007. The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. Genetics 176, 2223-2234.

Richeux, F., Cascante, M., Ennamany, R., Saboureau, D., Creppy, E.E., 1999. Cytotoxicity and genotoxicity of capsaicin in human neuroblastoma cells SHSY-5Y. Arch. Toxicol. 73, 403-409.

Rodriguez-Casado, A., 2014. The health potential of fruits and vegetables phytochemicals: notable examples. Crit. Rev. Food Sci., 00-00.

Romero-Jimenez, M., Campos-Sanchez, J., Analla, M., Muñoz-Serrano, A., Alonso-Moraga, Á., 2005. Genotoxicity and anti-genotoxicity of some traditional medicinal herbs. Mutat. Res. 585, 147-155.

Sánchez, A.M., Malagarie-Cazenave, S., Olea, N., Vara, D., Chiloeches, A., Díaz-Laviada, I., 2007. Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. Apoptosis 12, 2013-2024.

Scarborough, P., Nnoaham, K.E., Clarke, D., Capewell, S., Rayner, M., 2010. Modelling the impact of a healthy diet on cardiovascular disease and cancer mortality. J. Epidemiol. Commun. H., jech. 2010.114520.

Schalch, W., Cohn, W., Barker, F.M., Köpcke, W., Mellerio, J., Bird, A.C., Robson, A.G., Fitzke, F.F., van Kuijk, F.J., 2007. Xanthophyll accumulation in the human retina during supplementation with lutein or zeaxanthin–the LUXEA (LUtein Xanthophyll Eye Accumulation) study. Arch. Biochem. Biophys 458, 128-135.

Service, P., Hutchinson, E., MacKinley, M., Rose, M., 1985. Resistance to environmental stress in Drosophila melanogaster selected for postponed senescence. Physiol. Zool., 380-389.

Sim, K.H., Han, Y.S., 2007. The Antimutagenic and Antioxidant effects of red pepper seed and red pepper pericarp (Capsicum annuum L.). J. Food Sci. Nutr. 12, 273-278.

Soh, J.W., Hotic, S., Arking, R., 2007. Dietary restriction in Drosophila is dependent on mitochondrial efficiency and constrained by pre-existing extended longevity. Mech. Aging Dev. 128, 581-593.

Srinivasan, K., 2005. Spices as influencers of body metabolism: an overview of three decades of research. Food Res. Int. 38, 77-86.

Sun, T., Xu, Z., Wu, C.T., Janes, M., Prinyawiwatkul, W., No, H., 2007. Antioxidant activities of different colored sweet bell peppers (Capsicum annuum L.). J. Food Sci. 72, S98-S102.

Sun, X., Yang, X., Wang, E., 2005. Chromatographic and electrophoretic procedures for analyzing plant pigments of pharmacologically interests. Anal. Chim. Acta 547, 153-157.

Surh, Y.-J., Lee, S.S., 1995. Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. Life Sci. 56, 1845-1855.

Szallasi, A., Appendino, G., 2004. Vanilloid receptor TRPV1 antagonists as the next generation of painkillers. Are we putting the cart before the horse? J. Med. Chem. 47, 2717-2723.

Takahashi, E., Marczylo, T.H., Watanabe, T., Nagai, S., Hayatsu, H., Negishi, T., 2001. Preventive effects of anthraquinone food pigments on the DNA damage induced by carcinogens in Drosophila. Mutat. Res.- Fund. Mol. M. 480, 139-145.

Tasset-Cuevas, I., Fernández-Bedmar, Z., Lozano-Baena, M.D., Campos-Sánchez, J., de Haro-Bailón, A., Muñoz-Serrano, A., Alonso-Moraga, Á., 2013. Protective effect of borage seed oil and gamma linolenic acid on DNA: in vivo and in vitro studies. Plos One 8, e56986.

Topuz, A., Ozdemir, F., 2007. Assessment of carotenoids, capsaicinoids and ascorbic acid composition of some selected pepper cultivars (Capsicum annuum L.) grown in Turkey. J. Food Compos. Anal. 20, 596-602.

Trevithick-Sutton, C.C., Foote, C.S., Collins, M., Trevithick, J.R., 2006. The retinal carotenoids zeaxanthin and lutein scavenge superoxide and hydroxyl radicals: a chemiluminescence and ESR study. Mol Vis 12, 1127-1135.

Tsou, M.-F., Lu, H.-F., Chen, S.-C., Wu, L.-T., Chen, Y.-S., Kuo, H.-M., Lin, S.-S., Chung, J.-G., 2006. Involvement of Bax, Bcl-2, Ca2+ and caspase-3 in capsaicin-induced apoptosis of human leukemia HL-60 cells. Anticancer Res. 26, 1965-1971.

Tsuchiya, Y., Terao, M., Okano, K., Nakamura, K., Oyama, M., Ikegami, K., Yamamoto, M., 2011. Mutagenicity and mutagens of the red chili pepper as gallbladder cancer risk factor in Chilean women. Asian Pac. J. Cancer P. 12, 471-476.

Turner, D.R., Dreimanis, M., Holt, D., Firgaira, F.A., Morley, A.A., 2003. Mitotic recombination is an important mutational event following oxidative damage. Mutat. Res.- Fund. Mol. M. 522, 21-26.

Wang, M.-X., Jiao, J.-H., Li, Z.-Y., Liu, R.-R., Shi, Q., Ma, L., 2013. Lutein supplementation reduces plasma lipid peroxidation and C-reactive protein in healthy nonsmokers. Atherosclerosis 227, 380-385.

Wang, M., Tsao, R., Zhang, S., Dong, Z., Yang, R., Gong, J., Pei, Y., 2006. Antioxidant activity, mutagenicity/anti-mutagenicity, and clastogenicity/anti-clastogenicity of lutein from marigold flowers. Food Chem. Toxicol. 44, 1522-1529.

Wu, C.-C., Lin, J.-P., Yang, J.-S., Chou, S.-T., Chen, S.-C., Lin, Y.-T., Lin, H.-L., Chung, J.-G., 2006. Capsaicin induced cell cycle arrest and apoptosis in human esophagus epidermoid carcinoma CE 81T/VGH cells through the elevation of intracellular reactive oxygen species and Ca 2+ productions and caspase-3 activation. Mutat. Res.- Fund. Mol. M. 601, 71-82.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., Adler, P.N., 2008. The multiple-wing-hairs gene encodes a novel GBD–FH3 domain-containing protein that functions both prior to and after wing hair initiation. Genetics 180, 219-228.

Yang, K., Pyo, J., Kim, G.-Y., Yu, R., Ju, S., Kim, W., Kim, B.-S., 2009. Capsaicin induces apoptosis by generating reactive oxygen species and disrupting mitochondrial transmembrane potential in human colon cancer cell lines. Cell.. Mol. Biol. Lett. 14, 497-510.

Zhang, X., Zhao, W.-e., Hu, L., Zhao, L., Huang, J., 2011. Carotenoids inhibit proliferation and regulate expression of peroxisome proliferators-activated receptor gamma (PPARγ) in K562 cancer cells. Arch. Biochem. Biophys 512, 96-106.

Zhuang, Y., Chen, L., Sun, L., Cao, J., 2012. Bioactive characteristics and antioxidant activities of nine peppers. J. Funct. Foods 4, 331-338.

Zordan, M., Graf, U., Singer, D., Beltrame, C., Dalla Valle, L., Osti, M., Costa, R., Levis, A.G., 1991. The genotoxicity of nitrilotriacetic acid (NTA) in a somatic mutation and recombination test in Drosophila melanogaster. Mutat. Res. Lett. 262, 253-261.

CAPÍTULO III: Biological Activities of Two *Allium* species and Their Distinctive Organosulphur Compounds.

Chapter III

Artículo en preparación

ABSTRACT

The present research consists of an integrative evaluation on the health-beneficial properties of different doses of garlic (*Allium sativum*) and onion (*Allium cepa*) lyophilized extracts and their respective representative organosulfur compounds (diallyl disulfide, DADS; dipropyl disulfide, DPDS) using a multi-assay experimental design including animal and cellular models. We assessed their genotoxic, antigenotoxic and lifespan effect in an *in vivo* animal model (*Drosophila melanogaster)* as well as their pro-apoptotic capacities and clastogenic DNA activity against proliferative processes using an *in vitro* human cancer model (HL60 cell line). The results showed that (i) Garlic, onion, DADS and DPDS are non-genotoxic at all tested concentrations (0.625 and 5 mg/mL for the two *Allium sp.*, 0.17 and 1.37 mM for DADS, 0.04 and 1.33 mM for DPDS). (ii) All substances were antigenotoxic against H_2O_2-induced DNA damage with a positive dose-response effect and different inhibition percentages (33%-70% for onion, 19%-59% for garlic, 32%-46% for DPDS, 40%-95% for DADS). (iii) Daily intake of Allium sp. vegetables, DADS or DPDS had no positive effects on flies' lifespan at the assayed concentrations. Furthermore, the highest concentrations of garlic and DADS (5 mg/mL and 34 mM respectively) significantly decreased mean lifespan. In the same way, highest concentrations of tested substances, included as dietary supplement in the *Drosophila* food (onion: 2.5 mg/mL; garlic: 5 mg/mL; DPDS: 8, 16 and 33 mM; DADS: 17 and 34 mM) also significantly decreased the mean healthspan of the flies. (iv) Garlic and onion lyophilized extracts and DADS and DPDS exerted a cytotoxic effect on the HL60 promyelocytic cell growth

in a positive dose-dependent manner. Nevertheless the cytotoxic effect of onion was slight and could not reach the IC_{50} at the assayed concentrations. (v) Garlic and its representative organosulfur (DADS) exerted an internucleosomal DNA fragmentation as an index of induced proapoptotic activity on HL60 cells. (vi) Garlic, onion and DADS were able to significantly induce clastogenic strand breaks in theHL-60 measured by the comet assay.

It has been demonstrated the genomic safety of the assayed substances and their protective genetic effects against the oxidative mutagen hydrogen peroxide. Nevertheless, long-term treatments during the whole life of *Drosophila* are not beneficial for this animal model, only being healthy low-median concentrations. The chemopreventive activity of garlic on HL-60 cells could be due to its distinctive organosulphur DADS. Supplementary studies are needed to clarify the cell death pathway against garlic and DADS.

Key words: Garlic, onion, diallyl disulfide (DADS), dipropyl disulfide (DPDS), antigenotoxicity, lifespan, comet assay, *Drosophila*, HL-60 cells.

INTRODUCTION

Mediterranean diet is well known as one of the best nutritional patterns in human beings due to its beneficial effects on health. This diet, based in the high consumption of fruit, vegetables, wine and olive oil, with fish as the main animal protein contribution, is a prototype of a healthy and well balanced food intake (De Lorgeril and Salen, 2006). Today, most of the studies asserting these well-being effects agree that they may be due to its high antioxidant and phenolic contents (Trichopoulou and Vasilopoulou, 2000). Diet-derived antioxidants are implicated in maintaining a balanced homeostasis and scavenging of reactive oxygen species (ROS) as it is part of a highly effective defense network against oxidative stress which complements the endogenous defense enzymes (Ambrosone et al., 1999).

Garlic (*Allium sativum*) and onion (*Allium cepa*) are two diet native vegetables from Asia, widely used in different cultures and in traditional medicine for centuries (Rivlin, 2001). According to the FAO (2015), they are two of the most important vegetable crops with a worldwide production of 24,255,303 and 85,795,195 tons of garlic and onion respectively. In fact, their consumption has increased in recent years with the expansion of the Mediterranean and Asian cuisine. These vegetables have been linked to preventive effects against several diseases such as cancer, obesity, diabetes type-2, coronary heart disease and hypertension among others (Lanzotti, 2006; Sengupta et al., 2004; Yamamoto et al., 2005; Yoshinari et al., 2012). These properties were associated to their high content of thiosulfinates, a group of volatile organosulfur compounds originated from the decomposition of the allicin, which are also responsible for their typical

pungent aroma and taste (Cerella et al., 2011; Corzo-Martínez et al., 2007) (Amagase et al., 2001; Brodnitz et al., 1971). However, both vegetables showed a high variability among strains being diallyl sulfide (DAS), DADS and diallyl trisulfide (DATS) normally higher in garlic and dipropyl sulfide (DPS) and DPDS in onion (Shaath and Flores, 1998; Shukla and Kalra, 2007)

Oils and extracts of garlic were associated to several health-benefit activities. Among them, the protecting capacity against DNA damage induced by oxidative stress, their H_2O_2 scavenging activity and their ability to reduce the bioactivity of carcinogens and tumor cells proliferation (Benkeblia, 2005; Huang et al., 2015; Park et al., 2009; Tanaka et al., 2006). These capacities of extracts were directly linked to DADS, one of their major and most garlic distinctive constituents. This molecule was characterized as non-genotoxic, antigenotoxic and inhibitor of cell proliferation and pro-apoptotic in different cancer cell lines like leukaemia, colon, prostate, lung, bladder and skin (Gayathri et al., 2009; Jakubikova and Sedlak, 2005; Lin et al., 2008; Lu et al., 2004; Sundaram and Milner, 1996b; Tan et al., 2008; Wu et al., 2005; Yang et al., 2009)

On the other hand, onions are versatile vegetables that can be consumed fresh as well as in processed products. Like garlic extracts, they also showed a high oxy-radical scavenging capacity (Yang et al., 2004) and antigenotoxic effects (Aunanan and Kangsadalampai, 2010) and their ethanolic extracts and oils showed an antimutagenic activity (Ikken et al., 1999). In addition, onions extracts also decreased the viability and exerted an apoptotic effect in several cancer cell lines like HL60, MDA-MB-231, A549 and B16F10 (Seki et al., 2000; Shrivastava and Ganesh, 2010; Wang et

al., 2012; Wu et al., 2006). The pro-health properties of onions were widely related to DPDS, one of its most representative organosulfur compounds. This molecule showed a strong anticarcinogenic activity (Guyonnet et al., 2000a) and produced a protective effect towards DNA strand break and oxidative damage (Arranz et al., 2007a; Arranz et al., 2007b). Nevertheless, this compound had not antitumor effects in mice (Singh et al., 1998), did not decrease tumour cell growth and did not induce DNA-internucleosomal fragments on cancer cells (Garcia et al., 2009; Merhi et al., 2008; Singh et al., 1998; Sundaram and Milner, 1996b).

Hereby, we performed a qualitative and quantitative evaluation of the health-beneficial properties of garlic, onion and their representative organosulfur compounds (DADS and DPDS) in a multi-assay experimental design using animal and *in vitro* models. We assessed on their genotoxic, antigenotoxic and lifespan effects in *Drosophila melanogaster* flies, as 70 % of human disease genes are conserved in this organism (Richardson, 2015) and their proapoptotic capacities against cancer processes: cytotoxicity and clastogenic DNA activity in an *in vitro* human cancer model (HL60 cell line).

MATERIAL AND METHODS

Allium vegetables and single compounds

Two *Allium* species and two of its most distinctive organosulfur compounds were assayed. Garlic (*Allium sativum,* purple variety) and onion (*Allium cepa,* Victoria variety) were purchased in a local market. Thiosulfinates, DADS (CAS: 2179-57-9) from garlic and DPDS (CAS: 629-19-6) from onions, were purchased from Sigma (Cat numbers 317691 and 43550 respectively).

Preparation of the samples

Garlics and onions were washed twice with water, cut in slim slices, and freeze-dried at -80ºC. After that, both samples were lyophilized, pulverized with a mortar pestle, sieved and stored at 25 ºC in dark until use.

***In vivo* assays**

SMART test

Two *Drosophila melanogaster* fly strains carrying visible wing genetic markers were used in our experimental design: the flare (*flr*) strain *flr*^{3}*/ln (3LR) TM3, Bd*^{s} and the multiple wing-hair (*mwh*) strain *mwh/mwh*. The multiple wing hairs (*mwh, 3_0.3*) marker is a recessive viable mutation in homozygous flies, producing multiple-hairs trichomes in the fly adult body (Yan et al., 2008). The flare (*flr*^{3}*, 3_38.3*) marker is a homozygous recessive lethal mutation which produces malformed individual wing hairs in somatic cells of larvae. The *flr*^{3} allele is retained in a balancer chromosome carrying multiple inversions and a homozygous lethal dominant visible marker expressed in the edge wing (Ren et al., 2007).

Genotoxicity was determined using the somatic mutation and recombination test (SMART) as described by Graf et al. (1984) using pure water as negative control. The antigenotoxic activity was also determined using a modified SMART test following our standard protocols (Anter et al., 2011). Briefly, optimally virgin *flr*^{3}*/ln* (3LR) TM3, *ri p*^{p} *sep bx*^{34e} *e*^{s} *Bd*^{S} (flare) females were crossed with *mwh/mwh* strain males, obtaining 72 h transheterozygous F1larvae after an 8 h egg-laying on fresh yeast. Larvae were fed with *Drosophila* Instant Medium (Formula 4-24, Carolina

Biological Supply, Burlington, NC) in 4 mL vials. Genotoxicity assays consisted of eight experimental groups by supplementing the base larvae food (0.85 g) with different concentrations of onion (0.625 and 5 mg/mL), garlic (0.625 and 5 mg/mL), DADS (4 mM and 34 mM) and DPDS (4 mM and 33 mM). Single compounds concentrations were selected to mimic those described in the fresh *Allium sp*. (Block, 1992). Negative (distilled water) and positive (0.12M H_2O_2) concurrent controls were included. Antigenotoxicity experimental design was similar to the genotoxicity assays by concurrently treating the larvae with the tested substances supplemented with H_2O_2 (0.12M) as positive genotoxicant control. The emerged adults in each group were finally stored in 70 % ethanol until analysis.

Wings of twenty heterozygous flies (mwh/flr^3) of each compound and concentration were removed and mounted on slides with Faure´s solution (Arabic gum 50 g, glycerol 20 mL, chloral hydrate 50 g, distilled water 50 mL). Both dorsal and ventral surfaced were screened under a bright light photonic microscope at 400x magnification to detect small single spots (1-2 mwh or flr^3 cells), large single spots (3 or more cells) and twin spots (adjacent mwh and flr^3 cells). Single spots are produced by gene mutation, somatic recombination and deletion between the two markers. Twin spots are produced uniquely by recombination between the *flr^3* marker and the centromere.

In order to evaluate the genotoxic effect, the frequencies of total spots per wing of each series were statistically compared with the total spots of the negative control with the non-parametric U-test of Mann, Whitney and

Wilcoxon (Frei and Würgler, 1995). Antigenotoxicity was determined as the inhibition percentages (IP) using the total spots per wing determined at each concentration with the following formula (Abraham, 1994):

$$IP= [(a - b)/a] \times 100$$

where ***a*** represents the frequency of total spots induced by the treatment with genotoxine alone, and ***b*** represents the frequency of total spots obtained with genotoxine plus substance tested in the different combined treatments.

<u>Longevity assays</u>

All the longevity experiments were performed following our standard procedures (Fernández-Bedmar et al., 2011; Tasset-Cuevas et al., 2013). Transheterozygous larvae from a 12h egg-laying with the same genetic background described above were used in the life and health-span trials. Synchronized larvae of 72 ± 12 h were clustered in groups of 100 individuals in glass vials with 0.85 g of *Drosophila* Instant Medium in 4 mL of water solutions of the different experimental concentrations assayed (0.625, 1.25, 2.5 and 5 mg/mL for *Allium* vegetables; 4, 8, 16 and 33 mM for DPDS; 4, 8, 17 and 34 for DADS). The emerged flies were anesthetized under CO_2, separated into 10 single-sex groups, transferred to longevity vials and fed with the same treated medium during the whole experimental design. A concurrent treatment was also included using distilled water as negative control. The survivors were counted and the medium was renewed twice a week until all individuals die. Survival curves were plotted as estimated by the Kaplan-Meier method and the statistical

significance of curves were assessed using the Log-Rank (Mantel-Cox) method using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA).

***In vitro* assays**

<u>Cell line cultures and cytotoxicity assay</u>

In vitro assays were performed using the promyelotic leukemia HL60 cell line (kindly provided by Dr. Jose M. Villalba Montoro, University of Cordoba, Spain). Cells were passed twice a week (at 2.5×10^5 cells/mL) following our standard protocol (Anter et al., 2015) in complete RPMI 1640 medium (Biowhittaker, BE12-167F) containing 10% heat-inactivated foetal bovine serum (Biowhittaker, de14-801F), L-glutamine 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955) at 37ºC in a humidified atmosphere of 5% CO_2.

Cell viability was evaluated by Trypan blue exclusion assay. Cells (1×10^5 cells/mL) were seeded and incubated for 72 h in 96 well plates supplemented with 6 different concentrations *Allium species* (ranging from 0.002 mg/mL to 0.06 mg/mL) and 6 different concentrations of thiosulfinates (ranging from 0.0125 mM to 0.4 mM). A concurrent negative control (base medium without supplementation) was concurrently run. After incubation, Trypan blue was added to the cell suspension (1:1 ratio) and cells were counted in a Neubauer chamber under an inverted microscope at 100x magnification. Cell viability was expressed as percentage of survival with respect to control after 72 h period. IC_{50} values (concentration of tested compound causing 50% of cell growth inhibition)

were estimated for each treatment. Viability curves were plotted as mean viability ± Std of three independent replicas in each substance and concentration.

Internucleosomal DNA fragmentation assay

HL60 cells (1.5 x 10^6 cells/mL) were incubated with the same compounds and concentrations as in cytotoxicity assays for 5 h in 12-well plates. Thereafter cells were harvested, centrifuged at 2500 x g for 5 min. and washed with PBS. Total DNA was extracted using a commercial DNA-extraction kit (Blood Genomic DNA Extraction Mini Spin Kit, Canvax Biotech, Cordoba, Spain) according to the manufacturer instructions and subsequently treated with RNase overnight. DNA yielding was quantified in a Nanodrop™ (Thermo Scientific, Madrid, Spain). A total of 1500 ng of DNA per sample was loaded in a 2% agarose gel, stained with ethidium bromide and run by 120m at 60V. Internucleosomal DNA fragmentation was determined by the presence of a ladder band patterns with 200 bp multiple fragments.

Evaluation of DNA breakage ability: comet assay

The ability of the compounds to produce strand breaks in the DNA structure was determined by the alkaline comet assay as described Olive and Banáth (2006) with minor modifications. HL60 cells (5x10^5 cells) were plated in 1.5ml of culture medium supplemented with different concentrations of onion (0.004, 0.016 and 0.06 mg/mL), garlic (0.002, 0.004 and 0.008 mg/mL), DPDS (0.025, 0.1 and 0.4 mM) and DADS (0.01, 0.025 and 0.05 mM) and incubated for 5 h. After treatment, cells were washed

and adjusted to 6.25 x 10^5 cells/ml in PBS. Then, cells (1.6 x 10^4) were suspended in 75 µL pre-warmed low melting point agarose (A4018, Sigma) and 50 µL of the suspension were rapidly spread on microscope slides and covered with coverslips. After gelling for 30 min at RT, the coverslips was gently removed and the slides were put in a tank filled with lysis solution (2.5M NaCl, 100mM Na-EDTA, 10mM Tris, 250mM NaOH, 10% DMSO and 1% Triton X-100; pH= 13) at 4ºC for 1 h. Next, slides were removed from the lysis solution and incubated in alkaline electrophoresis buffer (300mM NaOH and 1mM Na-EDTA, pH= 13) at 4ºC for 20-30 min. Electrophoresis was then carried out in a fresh-made electrophoresis buffer for 15 minutes at 20 V and 400 mA in dark conditions. After electrophoresis, slices were gently washed in cold fresh-made neutralization buffer (0.4M Tris-HCl buffer, pH 7.5) for 10 min and allowed to dry overnight at RT in dark conditions. Finally, gels were stained with 7 µL propidium iodide, covered with a coverslip and photographed at X400 magnification in a Leica DM2500 epifluorescence microscope with a microscope. At least 50 cells were assessed for each treatment. Data were analysed using the Open Comet TM software (Gyori et al., 2014). The statistical ANOVA-Tukey test was applied (Serpeloni et al., 2008) using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA) in order to compare the results obtained for the different treatments and the negative control.

RESULTS

SMART test

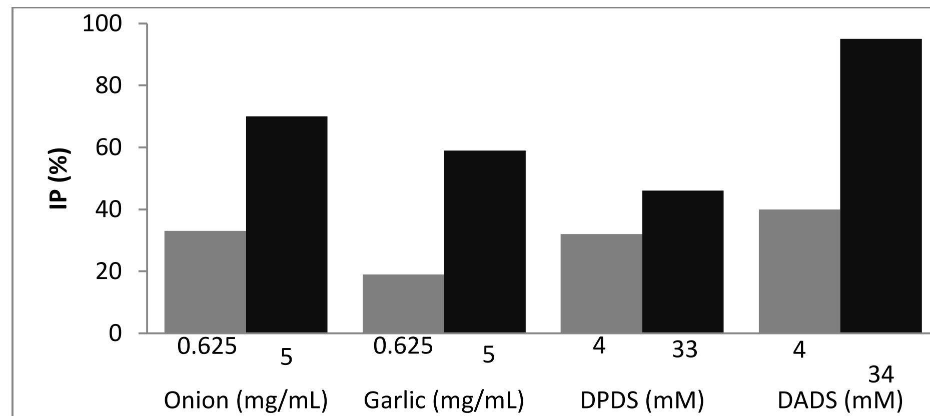
The results of genotoxicity and antigenotoxicity are shown in **Table 1**. All the assayed compounds were non genotoxic after larval feeding at all tested concentrations. Both *Allium* vegetables showed no differences compared with water control in single and total spots. Both concentrations of garlic and the higher concentration of DADS induced mutation rates even lower, but non-significant, than the negative control. Positive control (H_2O_2) resulted mutagenic as expected (0.37 total spots/wing) agreeing with our previous results (Fernández-Bedmar et al., 2011; Romero-Jimenez et al., 2005). The antigenotoxic potency of *Allium* sp. vegetables, DPDS and DADS against H_2O_2 exhibited a clear positive dose-response effect (**Figure 1**). The inhibition percentages values ranged from 33% and 70% for onion, 19% and 59% for garlic, 32% and 46% for DPDS, 40% and 95% for DADS.

Table1. Genotoxicity and antigenotoxicity results obtained in the SMART test in flies fed with different concentrations of the tested compounds in single and combined (with H_2O_2) treatments.

Compounds	N	Small spots (1-2 cells)	Large spots (>2 cells)	Twin spots	Total spots
Controls					
H_2O	40	0.10 (4)[a]	0	0	0.10 (4)
H_2O_2(0.12M)	40	0.30 (12)	0.05 (2)	0.02 (1)	0.37 (15)**
Onion (mg/mL*)*					
0.625	40	0.17 (7)	0	0	0.17 (7)**ns**
5	38	0.08 (3)	0.03(1)	0	0.10 (4)**ns**
0.625 + H_2O_2	40	0.20 (8)	0	0.05 (2)	0.25 (10)**ns**
5 + H_2O_2	38	0.08 (3)	0	0.03 (1)	0.11 (4)**ns**
Garlic (mg/mL)					
0.625	40	0.07 (3)	0	0	0.07 (3)**ns**
5	40	0.05 (2)	0	0	0.05 (2)**ns**
0.625 + H_2O_2	40	0.27 (11)	0	0.02 (1)	0.30 (12)*
5 + H_2O_2	40	0.15 (6)	0	0	0.15 (6)**ns**
DPDS (mM)					
4	40	0.22 (9)	0.02 (1)	0.02 (1)	0.27 (11)**ns**
33	40	0.07 (3)	0.07 (3)	0	0.15 (6)**ns**
4 + H_2O_2	40	0.20 (8)	0.05 (2)	0	0.25 (10)**ns**
33 + H_2O_2	40	0.17 (7)	0.02 (1)	0	0.20 (8)**ns**
DADS (mM)					
4	40	0.15 (6)	0	0	0.15 (6)**ns**
34	26	0.04 (1)	0	0	0.04 (1)**ns**
4 + H_2O_2	40	0.20 (8)	0.02 (1)	0	0.22 (9)**ns**
34 + H_2O_2	40	0.02 (1)	0	0	0.02 (1)**ns**

a: number of spots per wing, N: number of wings; ns: non-significant (p>.005), *: Statistically significant compared with the control (p≤0.05), **: highly significant compared with the control (p≤0.01). (non-parametric U test of Mann, Whitney and Wilcoxon; (Frei and Würgler, 1995).

Figure 1 Mutagenicity inhibition percentages produced by the tested compounds against H_2O_2 –DNA induced damage (*Drosophila melanogaster* model).



Longevity assays

Flies' survival curves for all treatments are plotted in **Figure 2**. *Allium* species, DADS and DPDS had no positive effects on flies' lifespan. In general, a lifespan reduction was observed in all the tested compounds, but results were significantly lower at high supplementation levels. In onions, only two higher concentrations (2.5 and 5 mg/mL) decreased the mean lifespan by 13 and 8% respectively, but only highest concentration was significant (**Table 2**). Flies fed with DPDS, onion most distinctive active compound, decreased the lifespan in a dose-dependent manner, but only supplementation with 8, 16 and 33 mM were significant. On the other side, garlic and DADS showed a similar effect, being only the highest supplementation (5 mg /mL and 34 mM) the only one that significantly decreased the flies´ lifespan. Healthspan is the healthy adult period of unimpaired life that precedes functional decline (Lee et al., 2010). It is

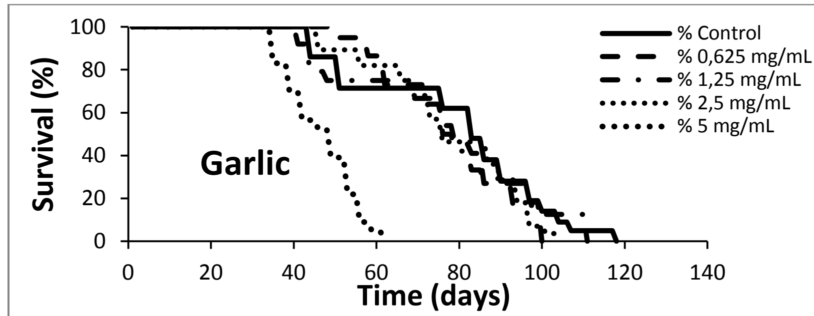
Table 2. Effects of the tested compounds at different concentrations on the *Drosophila melanogaster* mean lifespan and healthspan.

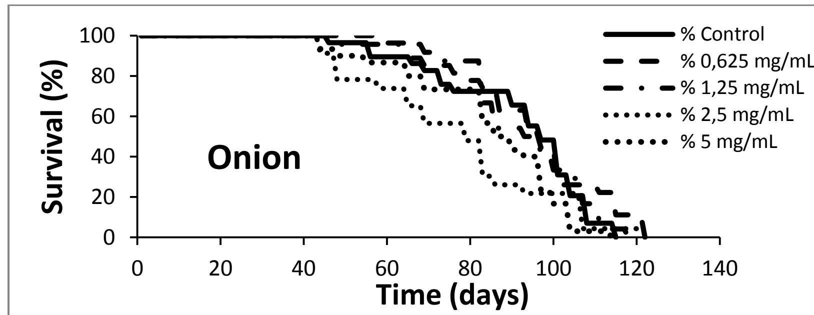
	Mean lifespan (days)	Mean lifespan difference (%)[a]	Health-span (75th percentile) (days)	Health-span difference (%)[a]
Onion (mg/mL)				
Control	92.24±3.58	0	64.37±3.75	0
0.625	95.77±3.45	4	71.29±3.26	11
1.25	92.83±3.36	1	73.00±5.60	13
2.5	80.31±4.69	-13	50.71±2.97*	-21
5	84.47±3.48*	-8	57.37±3.87	-12
Garlic (mg/mL)				
Control	81.25±4.57	0	51.14±4.31	0
0.625	79.51±3.30	-2	58.83±1.76	15
1.25	76.21±4.46	-6	47.29±3.73	-7
2.5	77.68±3.35	-4	53.57±3.48	5
5	46.83±1.78***	-42	36.30±0.843***	-29
DPDS (mM)				
Control	91.63±2.06	0	77.37±1.05	0
4	89.58±2.39	-2	73.00±2.80	-6
8	84.23±2.38*	-8	67.12±2.69**	-13
16	82.25±3.23*	-10	64.20±6.63*	-17
33	78.38±3.52*	-14	52.12±4.02***	-33
DADS (mM)				
Control	88.00±2.26	0	73.11±2.40	0
4	84.64±3.01	-4	68.00±3.34	-7
8	88.83±2.36	1	75.22±2.50	3
17	86.21±3.27	-2	62.87±3.96*	-14
34	59.78±5.45***	-32	40.00±2.00***	-45

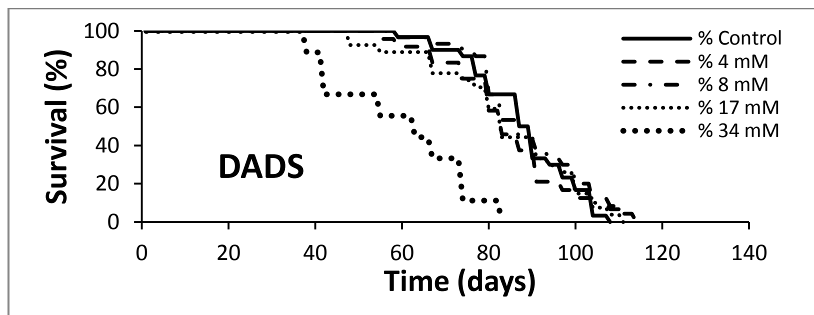
[a] Difference between treated flies and the concurrent negative control (water) in percentage. Positive results indicate that lifespan was increased and negative results indicate that lifespan was decreased. Statistical significance: * = p ≤0.05, ** = p ≤0.01, *** = p ≤ 0.001 (log-Mantel-Cox test)

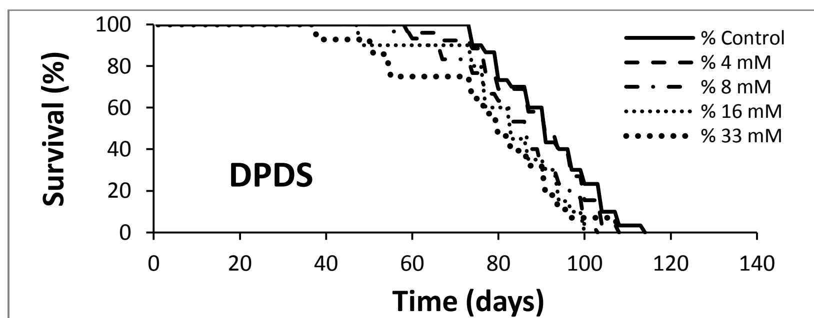
1

Figure 2. Effect of the tested compounds in the life span of *Drosophila melanogaster* flies fed with the tested compounds.









important to consider the quality of a prolonged life and for this reason healthspan is a new focus in aging research. Like lifespan indexes, healthspan was also adversely affected by all tested compounds. The higher concentrations of the tested substances included in the *Drosophila* supplemented diets (onion: 2.5 mg/mL; garlic: 5 mg/mL; DPDS: 8, 16 and 33 mM; DADS: 17 and 34 mM) significantly decreased the mean healthspan. DPDS decreased the mean healthspan in a dose-dependent manner (**Table 2**). It is noteworthy that there is a general agreement between lifespan and healthspan results.

Cytotoxicity and proapoptotic assays

The cytotoxic effects of *Allium* vegetables and their distinctive compounds (DADS and DPDS) on the survival of HL60 cells are shown in **Figure 3**. Garlic and DADS exerted a cytotoxic effect on cell growth in a positive dose-dependent manner after 72 h of incubation, with IC_{50} of 0.003 mg/mL and 0.06 mM respectively. Nevertheless onion cytotoxic effect on HL60 cell was slight, reaching only a 30% of grow inhibition. DPDS showed to be cytotoxic with a high IC_{50} (0.25 mM).

Figure 4 shows the results of proapoptotic effects of different concentrations of garlic, onion, DADS and DPDS in HL60 cells measured as internucleosomic programmed fragmentation (Masuda et al., 2015). DNA fragmentation was observed at all tested concentrations of garlic (0.002-0.0625 mg/mL) in a dose-depend manner and at the highest concentrations of DADS (0.1, 0.2 and 0.4 mM). Nevertheless, no DNA

internucleosomal fragments were induced neither by onion nor by DPDS at the assayed concentrations.

Figure 3 Viability of HL60 cells treated during 72 hours with different concentrations of onion, garlic and their respective organosulfur compound, DPDS and DADS. Curves are plotted as mean percentages with respect to the control (three independent replicates).

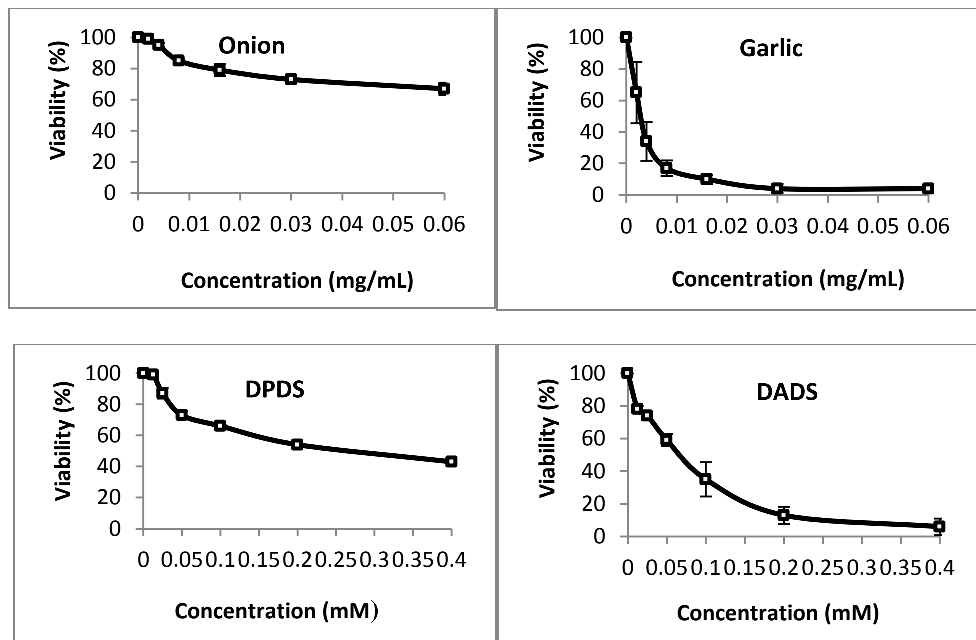
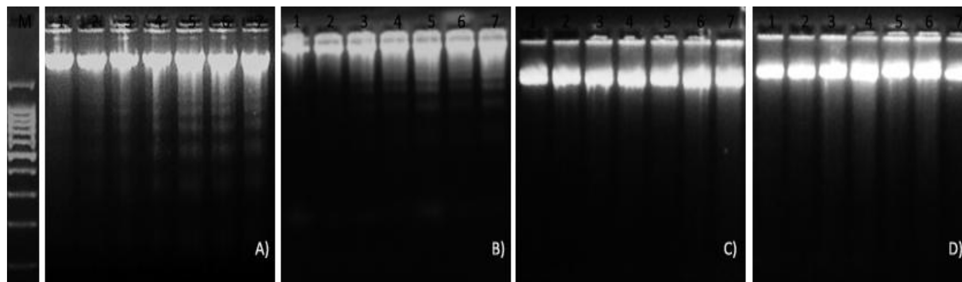


Figure 4. Internucleosomal DNA fragmentation of HL60 cells after 5 h-treatments with different concentrations of garlic, DADS, onion and DPDS.

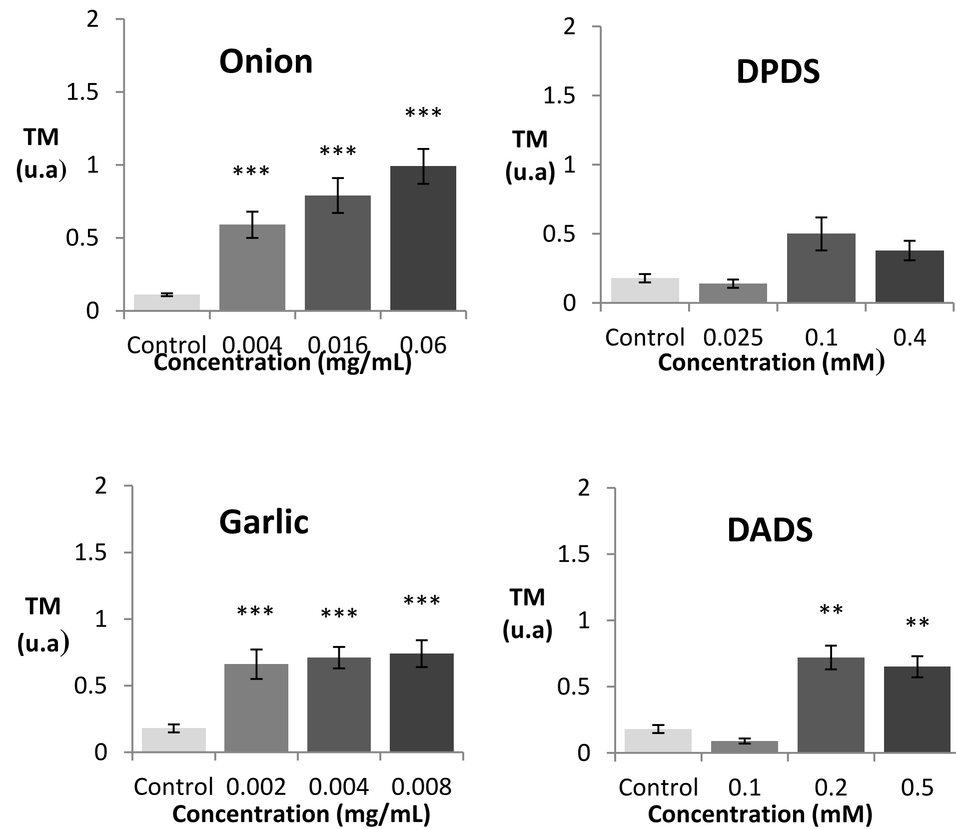


Garlic (A): Control (lane 1), 0.002 mg/mL (lane 2), 0.004 mg/mL (lane 3), 0.008 mg/mL (lane 4), 0.016 mg/mL (lane 5), 0.03 mg/mL (lane 6), 0.06 mg/mL (lane 7). DADS (B): Control (lane 1), 0.0125 mM (lane 2), 0.025 mM (lane 3), 0.05 mM (lane 4), 0.1 mM (lane 5), 0.2 mM (lane 6), 0.4 mM (lane 7). Onion (C): Control (lane 1), 0.002 mg/mL (lane 2), 0.004 mg/mL (lane 3), 0.008 mg/mL (lane 4), 0.016 mg/mL (lane 5), 0.03 mg/mL (lane 6), 0.06 mg/mL (lane 7). DPDS (D): Control (lane 1), 0.0125 mM (lane 2), 0.025 mM (lane 3), 0.05 mM (lane 4), 0.1 mM (lane 5), 0.2 mM (lane 6), 0.4 mM (lane 7).

DNA single strand breaks

Both vegetables induced a significant ($p \leq 0.001$) increase in the TM parameter at all tested concentrations. On the contrary, only DADS -garlic most important organosulfur- was able to induce a significant ($p \leq 0.01$) increase of this parameter at 28 and 56 µM (**Figure 5**).

Figure 5. HL 60 DNA integrity after 5 h-treatment with different concentrations of the tested compounds. Data is expressed as TM parameter (comet assay; (Olive and Banáth, 2006); mean value of three independent replicates). Statitiscall significance compared with negative control: *** = p ≤ 0.000, ** = p ≤ 0.01 and * = p ≤ 0.05.



DISCUSSION

Garlics and onions have been traditionally used as food sources around the world over the centuries. Their health benefits, such as the prevention of cardiovascular diseases, cancer and even aging, probably helped to their

widespread and maintenance in different cuisines and countries (Corzo-Martínez et al., 2007). Despite their popularity there are scarce systematic, integrate and multifocal studies assessing the genotoxicity, antigenotoxicity, and health-span effects as well as their cytotoxicity and pro-apoptotic properties against cancer cells of these vegetables and even less assessing their distinctive organosulfur compounds (DADS and DPDS.

In vivo studies at the DNA and individual level (genotoxicity, antigenotoxicity and longevity) were carried out using *Drosophila melanogaster* flies. This specimens are widely used as a genetic animal model due to its homology with several mammal models in biological, physiological and neurological traits (Jones and Grotewiel, 2011; Reiter et al., 2001). It was demonstrated that more than 70 % of human disease-causing genes have a functional homolog in this fly model, and it was also largely used to evaluate the genotoxicity of different compounds due to its accuracy (Mukhopadhyay et al., 2004; Siddique et al., 2005). Carcinogen and chemopreventive molecules present in the food should be taken into account and evaluated in every complex mixture destined to alimentation. Genotoxic assays are considered as the first step in the screening of these components. Our vegetables were previously determined as a non-mutagenic in *S. thyphimurium* (Ames assay) (Martínez et al., 1999). *Drosophila* wing spot test is an ideal system to evaluate complex mixtures like foods. To our knowledge, this is the first time to characterize the genotoxic effect of garlic, onion and two representative organosulfur present in their matrixes (DADS and DPDS respectively) using *Drosophila* model. Garlic was non-genotoxic at the assayed concentrations compared

to control, as it was also previously described by Shukla and Taneja (2002) and Abraham and Kesavan (1984). In these studies was demonstrated that aqueous garlic extracts (5% v/v) and fine garlic powder (7.5, 5 and 2.5 g/Kg body weight) supplementation did not induced chromosomal aberrations or DNA damage in mouse bone marrow cells. Same results were obtained by Sowjanya et al. (2009) at 3, 6 and 12 mg/culture in human lymphocytes and by Chughtai et al. (1998) (extracts of fresh garlic bulbs) in yeast. In the case of onion, non-genotoxicity was found at the tested concentrations (0.625 and 5 mg/mL). These results are in agreement with those obtained by Kulkarni et al. (2010) in several *Salmonella* strains. In the same way, DPDS and DADS, the active principles of garlic and onion, were non-genotoxic in our SMART trials. Nevertheless, previous reports showed different results. As example, Musk et al. (1997) demonstrated that DADS induced both chromosome aberrations and sister chromatid exchanges (genotoxic effects) exhibiting activity at concentrations below 0.07 mM in a Chinese hamster ovary cell line. However, this controversy could be partially explained due to methodological differences and the concentrations tested. Controversial results are commonly found for a single molecule when tested in different assays and *in vivo* carcinogenic trials are needed.

Despite the fact that onions are widely employed in the human diet, the number of genotoxicity studies carried out of DPDS is scarce being the first time to test the genotoxic and antigenotoxic effects of this compound using *in vivo* models (Guyonnet et al., 2000b, 2001b). The lack of genotoxicity activity in *Allium* vegetables, DADS and DPDS at assayed

concentrations in the *Drosophila* eukaryotic model confirm their safe use as no controversial results are available.

One of the strategies for coping with the genotoxic carcinogens, is to identify effective antimutagens and anticarcinogens in order to increase man´s exposure to them as a way for decreasing the cancer incidence (Ramel et al., 1986). This is the second step in the search of real nutraceutic substances. In our case, antigenotoxicity assays were conducted using hydrogen peroxide as positive genotoxicant model since this compound is able to induce somatic mutation and mitotic recombination in *Drosophila melanogaster* (Romero-Jimenez et al., 2005) and several well documented effects in the DNA integrity and stability.

Desmutagenic activity of garlic and different types of garlic extracts was described previously in several induced mutagenesis models. It was demonstrated that garlic water garlic protected mice strains against the genotoxic effects of gamma-radiation (Singh et al., 1996) and also exerted different antimutagenic effects in mice by modulating the genotoxicity induced by cyclophosphamide human lymphocytes in a dose dependent manner (Shukla and Taneja, 2002; Sowjanya et al., 2009). In the same way, methanolic and ethanolic garlic extracts prepared by different processing methods (raw, grilled, pickled), showed inhibitory activities on H_2O_2-induced DNA damage in human leukocytes using the comet assay (Kim et al., 2010) and reduced the chromosomal aberrations rate induced by DMBA in mice bone marrow (Sengupta et al., 2002). In flies, raw garlic methanolic extracts reduced the urethane mutagenicity in both standard and high bioactivation *Drosophila melanogaster* crosses (Aunanan and

Kangsadalampai, 2010). Our results are in agreement, demonstrating that garlic behaves as an important antigenotoxine. This activity was probably due to the DADS content, since it inhibited the 95% of the mutagenic damage induced by H_2O_2 when it was tested acting as a simple molecule. This was proposed previously in several reports indicating the protective effect of DADS against mutagenic substances such as BPDE ((+)-anti-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene), SO (styrene oxide) and and 4-NQO (Guyonnet et al., 2001a), aflatoxin B1 (AFB1) and N-nitrosodimethylamine (NDMA) in rat livers (Le Bon et al., 1997) and NMDA and NPYR in HepG2 cells (Arranz et al., 2007b).

Similar results were reported on the desmutagenic activity of onions. Ethanolic extracts showed a strong inhibitory effect against NDBA (Ames test) (Ikken et al., 1999) and Welsh onion juice suppressed the mutagenic activity of BaP and 4QNO and reduced the number of DMBA-induced chromosome aberrations in bone marrow cells of rats (Ito et al., 1986). Furthermore, onion supplementation protected *Drosophila melanogaster* flies against urethane-induced DNA damage (Aunanan and Kangsadalampai, 2010). Our findings agree with those reports since onion supplementation reduced the mutagenic effects of H_2O_2 near to 65% at the higher concentration tested. In the same way, DPDS, showed an important desmutagenic activity when it was tested as an individual molecule. But unlike the results obtained in garlic, its effect was lower acting as a single molecule than the assessed in onions. In this sense, DPDS strongly increased dimethyl nitrosamine (DMN) mutagenicity in *Salmonella typhimurium* (Guyonnet et al., 2000a) and reduced NPYR/NDMA-induced

oxidative DNA damage in HepG2 cells at 5 µM (Arranz et al., 2007b). Our DPDS antigenotoxic results are in agreement with those obtained by other authors. In our study, we demonstrated that *Allium* vegetables have a protective role against H_2O_2 induced damage using the *Drosophila melanogaster* animal model for the first time. This effect could probably be due to the ant oxidative properties its well-known scavenging potential against free-radicals of their respective organosulfur compounds (Benkeblia, 2005; Imai et al., 1994; Prakash et al., 2007), since same results were observed in the vegetables and simple molecule assessments.

Longevity assays are one of most simple and efficient methodological approaches to evaluate the aging and anti-aging capabilities of simple compounds and complex mixtures of higher organisms. *Drosophila melanogaster* is considered an very useful genetic model on aging research since its similarities with humans metabolic pathways controlling nutrient uptake, storage and metabolism (Anh et al., 2011; Zhang et al., 2014). In addition, this model has a short lifespan compared with similar *in vivo* models, reducing the experimental periods.

To our knowledge, this is the first assessment on the effect of onions, DADS and DPDS on the *D. melanogaster* lifespan and one of the few existent assessing this effect in garlics (Lei et al. (2014). Both vegetables and their compounds significantly decreased the flies´ mean lifespan at their highest concentrations, as well as at lower concentrations in DADS. Our results fit with the hypothesis that individual organosulfur compounds are responsible for the reduced viability of the insects, since results between vegetables and their compounds are similar. However, previous reports

showed beneficial effects of garlic extracts on animal lifespan, including *D. melanogaster* and *C. elegans* (Huang et al. (2015). Those differences could be explained since raw garlic was used in our study and previous reports evaluated garlic extracts. In this sense, Prowse et al. (2006), demonstrated that garlic juice had insecticidal activity across life stages of flies at a wide range of concentrations (0.25-5 %) in two dipteran pests (*Delia radicum* and *Musca domestica*). It is noticeable that high doses are only used for medicinal purposes in acute treatments in humans (Penner et al., 2005). Hence, high doses of garlic would not be advisable for long term treatments purposes. Nutraceuticals or dietary supplements tend to deliver the bioactive compounds at dosages that exceed what could be naturally obtained from foods. These results agree with the fact that similar effects on lifespan were caused by garlic, onion, DADS and DPDS in our *D. melanogaster* experiments, being these effects more marked in garlic and DADS treatments.

Our results showed that only garlic and DADS have a strong cytotoxic effect and induce a clear DNA pro-apoptotic internucleosomal fragmentation against HL60 cells. Previous reports demonstrated that garlic and DADS exerted a chemopreventive effect through different pathways: (i) by increasing apoptosis and *Bcl-2* expression and decreasing p53 protein and *Bax* expression in lung cancer cells (NCI-H1299)(Hong et al., 2000); (ii) by increasing intracellular ROS in A549 cells (Wu et al., 2005); (iii) by inhibiting cell proliferation in CaCo-2 and HT-29 cells repressing histone deacetylase activity and histone hyperacetylation and increasing the p21(waf1/cip1) expression (Druesne et al., 2004) and (iv) by inducing apoptosis through

activation of caspase-3 expression in HL60 (Kwon et al., 2002). In addition, Yang et al. (2009), observed that supplementation of different DADS concentrations (0.5, 10 and 25 µM) had a proapoptotic effect in COLO 205 cell line through induction of reactive oxygen species and caspase cascade. On the contrary, the molecular mechanism for the cytotoxic effect of onion and DPDS is relatively weak and less explained which fits with our results. In this sense, Sundaram and Milner (1996a) demonstrated that DPDS (100 µM) was an inefficient molecule to inhibit the cell growth and to induce programmed cell death in tumour cells (HCT-15). However, Wu et al. (2006) suggested that onion oil induces cell cycle arrest and apoptosis through ROS production in A549 cells. Our studies performed evaluating the properties of raw onion samples and DADS confirmed that their chemopreventive properties are weak despite type of sample employed.

In this sense, it was been proposed that the carcinogenic inhibition mechanism of DADS is mediated through a modulation of cytochrome P450–dependent monooxygenases and/or the acceleration of carcinogen detoxification by upregulating phase II-enzymes (Chun et al., 2001; Iciek et al., 2009).

DNA internucleosomal fragmentation is believed a hallmark of apoptosis, although his alone should not be considered as a criterion for assessing apoptotic cell death (Cohen et al., 1992). The mutagens can induce single and double strand breaks and play an important role in the pathogenesis of cancer (Ames, 1989; Halliwell and Aruoma, 1991). In order to test the

ability of the tested substances to induce DNA breaks in HL60 cells, it was used the single cell gel electrophoresis assay, also known as “comet test”. For this reason we performed for the first time a study of DNA-damage induced by garlic, onion, DADS and DAPS using the alkaline “comet assay” in HL60 leukemic cells as a complementary test to quantify the extent of the DNA damage induced by our compounds (Olive and Banáth, 2006). Single cell electrophoresis gel (comet) has been used widely to detect natural substances which are able to induce DNA damage in cancer cells (Balasenthil et al., 2002; Cardile et al., 2002), being the tail moment (TM) an accurate parameter to quantify the DNA migration and thus, the DNA fragmentation status (Olive and Banáth, 2006). With this parameter we differentiated apoptosis-induced from necrosis-induced DNA damage (a TM>30 is considered as an indicator of apoptosis and a TM between 5 and 30 is considered as a necrotic process (Fairbairn and O’Neill, 1995)).

Our results showed that garlic and onion induced DNA damage in HL60 by necrosis (TM <2). These results are agreement with the results obtained in cytotoxicity and DNA-fragmentation assays.

Arranz et al. (2007b) assaying higher concentrations of DADS (>5 µM), showed DNA damage in HepG2 cells in the alkaline comet assay. However, controversial results were also reported by (Belloir et al., 2006), suggesting that DADS was not genotoxic at concentrations between 5-100 µM in the same *in vitro* model.

Our results with DPDS disagree with those obtained by Arranz et al. (2007b), who also showed that DPDS could act in a positive dose-

dependent manner since the higher concentrations tested (>5 µM) caused DNA damage in HepG2 cells (data not shown) by comet assay.

In conclusion, our experimental results provide the evidence that (i) garlic, onion DADS and DPDS are safe and antigenotoxic against oxidative mutagens in a dose dependent manner. (ii) The decrease of lifespan in some highest concentrations of all compounds is a confirmation that in long term consumption of our tested compounds are safe only at low concentrations in a *Drosophila* model. (iii) Garlic exerted a clear chemopreventive activity, being its distinctive organosulphur DADS the most probable cause of such as activities. (iv): The slight cytotoxic effect of onion is probably mediated by non-apoptotic mechanism and (v) supplementary studies are necessary to clarify the cell death pathway against garlic and DADS

REFERENCES

Abraham, S.K., 1994. Antigenotoxicity of coffee in the Drosophila assay for somatic mutation and recombination. Mutagenesis 9, 383-386.

Abraham, S.K., Kesavan, P., 1984. Genotoxicity of garlic, turmeric and asafoetida in mice. Mutat. Res. 136, 85-88.

Amagase, H., Petesch, B.L., Matsuura, H., Kasuga, S., Itakura, Y., 2001. Intake of garlic and its bioactive components. J. Nutr. 131, 955S-962S.

Ambrosone, C.B., Freudenheim, J.L., Thompson, P.A., Bowman, E., Vena, J.E., Marshall, J.R., Graham, S., Laughlin, R., Nemoto, T., Shields, P.G., 1999. Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. Cancer Res. 59, 602-606.

Ames, B.N., 1989. Endogenous oxidative DNA damage, aging, and cancer. Free Radical Res. 7, 121-128.

Anh, N.T.T., Nishitani, M., Harada, S., Yamaguchi, M., Kamei, K., 2011. A Drosophila model for the screening of bioavailable NADPH oxidase inhibitors and antioxidants. Mol. Cell Biochem. 352, 91-98.

Anter, J., Demyda-Peyras, S., Delgado-Torre, M.P., Campos-Sanchez, J., Luque De Castro, M.D., Muñoz-Serrano, A., Alonso-Moraga, Á., 2015. Biological and health promoting activity of vinification byproducts produced in Spanish vineyards. S. Afr. J. Enol. Vitic. 36, 126-133.

Anter, J., Fernández-Bedmar, Z., Villatoro-Pulido, M., Demyda-Peyras, S., Moreno-Millán, M., Alonso-Moraga, Á., Muñoz-Serrano, A., Luque de Castro, M.D., 2011. A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 723, 165-170.

Arranz, N., Haza, A., Garcia, A., Delgado, E., Rafter, J., Morales, P., 2007a. Effects of organosulfurs, isothiocyanates and vitamin C towards hydrogen peroxide-induced oxidative DNA damage (strand breaks and oxidized purines/pyrimidines) in human hepatoma cells. Chem.-Biol. Interact. 169, 63-71.

Arranz, N., Haza, A., Garcia, A., Möller, L., Rafter, J., Morales, P., 2007b. Protective effects of organosulfur compounds towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Food Chem. Toxicol. 45, 1662-1669.

Aunanan, A., Kangsadalampai, K., 2010. Effect of Preparation and Temperature treatments on Antimutagenicity against Urethane in Drosophila melanogaster and Antioxidant activity of Three Allium members. Thai J. Toxicol. 23, 108-116.

Balasenthil, S., Rao, K., Nagini, S., 2002. Garlic induces apoptosis during 7, 12-dimethylbenz [a] anthracene-induced hamster buccal pouch carcinogenesis. Oral Oncol. 38, 431-436.

Belloir, C., Singh, V., Daurat, C., Siess, M., Le Bon, A., 2006. Protective effects of garlic sulfur compounds against DNA damage induced by direct- and indirect-acting genotoxic agents in HepG2 cells. Food Chem. Toxicol. 44, 827-834.

Benkeblia, N., 2005. Free-radical scavenging capacity and antioxidant properties of some selected onions (Allium cepa L.) and garlic (Allium sativum L.) extracts. Braz. Arch. Biol. Techn. 48, 753-759.

Block, E., 1992. The Organosulfur Chemistry of the Genus AZZium-Implications for the Organic Chemistry of Sulfur. Angav.

Brodnitz, M.H., Pascale, J.V., Van Derslice, L., 1971. Flavor components of garlic extract. J. Agr. Food Chem. 19, 273-275.

Cardile, V., Scifo, C., Russo, A., Falsaperla, M., Morgia, G., Motta, M., Renis, M., Imbriani, E., Silvestre, G., 2002. Involvement of HSP70 in resveratrol-induced apoptosis of human prostate cancer. Anticancer Res. 23, 4921-4926.

Cerella, C., Dicato, M., Jacob, C., Diederich, M., 2011. Chemical properties and mechanisms determining the anti-cancer action of garlic-derived organic sulfur compounds. Anti-Cancer Agent. Me. 11, 267-271.

Cohen, G., Sun, X., Snowden, R., Dinsdale, D., Skilleter, D., 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem. J. 286, 331-334.

Corzo-Martínez, M., Corzo, N., Villamiel, M., 2007. Biological properties of onions and garlic. Trends Food Sci. Tech. 18, 609-625.

Chughtai, S.R., Dhmad, M., Khalid, N., Mohamed, A., 1998. Genotoxicity testing of some spices in diploid yeast. Pakisthan J. Bot 30, 33-38.

Chun, H.S., Kim, H.J., Choi, E.H., 2001. Modulation of cytochrome P4501-mediated bioactivation of benzo [a] pyrene by volatile allyl sulfides in human hepatoma cells. Biosci. Biotech. Bioch. 65, 2205-2212.

De Lorgeril, M., Salen, P., 2006. The Mediterranean-style diet for the prevention of cardiovascular diseases. Public Health Nutr. 9, 118-123.

Druesne, N., Pagniez, A., Mayeur, C., Thomas, M., Cherbuy, C., Duée, P.-H., Martel, P., Chaumontet, C., 2004. Diallyl disulfide (DADS) increases histone acetylation and p21waf1/cip1 expression in human colon tumor cell lines. Carcinogenesis 25, 1227-1236.

Fairbairn, D.W., O'Neill, K.L., 1995. Necrotic DNA degradation mimics apoptotic nucleosomal fragmentation comet tail length. In Vitro Cell.Dev.-An. 31, 171-173.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Frei, H., Würgler, F.E., 1995. Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in Drosophila. Mutat. Res. 334, 247-258.

Garcia, A., Morales, P., Arranz, N., Delgado, M.E., Rafter, J., Haza, A.I., 2009. Antiapoptotic effects of dietary antioxidants towards N-nitrosopiperidine and N-nitrosodibutylamine-induced apoptosis in HL-60 and HepG2 cells. J. Appl. Toxicol. 29, 403-413.

Gayathri, R., Gunadharini, D.N., Arunkumar, A., Senthilkumar, K., Krishnamoorthy, G., Banudevi, S., Vignesh, R.C., Arunakaran, J., 2009. Effects of diallyl disulfide (DADS) on expression of apoptosis associated proteins in androgen independent human prostate cancer cells (PC-3). Mol. Cell Biochem. 320, 197-203.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.-H., Le Bon, A.-M., 2000a. Liver subcellular fractions from rats treated by organosulfur compounds from Allium modulate mutagen activation. Mutat. Res. 466, 17-26.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.-H., Le Bon, A.-M., 2001a. Antimutagenic activity of organosulfur compounds from Allium is associated with phase II enzyme induction. Mutat. Res. 495, 135-145.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2000b. Liver subcellular fractions from rats treated by organosulfur compounds

from Allium modulate mutagen activation. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 466, 17-26.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2001b. Antimutagenic activity of organosulfur compounds from Allium is associated with phase II enzyme induction. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 495, 135-145.

Gyori, B.M., Venkatachalam, G., Thiagarajan, P.S., Hsu, D., Clement, M.V., 2014. OpenComet: An automated tool for comet assay image analysis. Redox Biol. 2, 457-465.

Halliwell, B., Aruoma, O.I., 1991. DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. FEBS Lett. 281, 9-19.

Hong, Y.-S., Ham, Y.-A., Choi, J.-H., Kim, J., 2000. Effects of allyl sulfur compounds and garlic extract on the expressions of Bcl-2, Bax, and p53 in non small cell lung cancer cell lines. Exp. Mol. Med. 32, 127-134.

Huang, C.-H., Hsu, F.-Y., Wu, Y.-H., Zhong, L., Tseng, M.-Y., Kuo, C.-J., Hsu, A.-L., Liang, S.-S., Chiou, S.-H., 2015. Analysis of lifespan-promoting effect of garlic extract by an integrated metabolo-proteomics approach. J. Nutr. Biochem.

Iciek, M., Kwiecien, I., Wlodek, L., 2009. Biological properties of garlic and garlic-derived organosulfur compounds. Environ. Mol. Mutagen. 50, 247-265.

Ikken, Y., Morales, P., Martínez, A., Marín, M.L., Haza, A.I., Cambero, M.I., 1999. Antimutagenic effect of fruit and vegetable ethanolic extracts against N-nitrosamines evaluated by the Ames test. J. Agr. Food Chem. 47, 3257-3264.

Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H., Itakura, Y., 1994. Antioxidant and radical scavenging effects of aged garlic extract and its constituents. Planta Med. 60, 417-420.

Ito, Y., Maeda, S., Sugiyama, T., 1986. Suppression of 7, 12-dimethylbenz [a] anthracene-induced chromosome aberrations in rat bone marrow cells by vegetable juices. Mutat. Res. 172, 55-60.

Jakubikova, J., Sedlak, J., 2005. Garlic-derived organosulfides induce cytotoxicity, apoptosis, cell cycle arrest and oxidative stress in human colon carcinoma cell lines. Neoplasma 53, 191-199.

Jones, M.A., Grotewiel, M., 2011. Drosophila as a model for age-related impairment in locomotor and other behaviors. Exp. Gerontol. 46, 320-325.

Kim, J.-M., Jeon, G.-I., Park, E.-J., 2010. Protective Effect of Garlic (Allium sativum L.) Extracts Prepared by Different Processing Methods on DNA Damage in Human Leukocytes. J. Korean Soc. Food Sci. Nutr. 39, 805-812.

Kulkarni, M., Ascough, G., Verschaeve, L., Baeten, K., Arruda, M., Van Staden, J., 2010. Effect of smoke-water and a smoke-isolated butenolide on the growth and genotoxicity of commercial onion. Sci. Hortic. Amsterdam 124, 434-439.

Kwon, K.-B., Yoo, S.-J., Ryu, D.-G., Yang, J.-Y., Rho, H.-W., Kim, J.-S., Park, J.-W., Kim, H.-R., Park, B.-H., 2002. Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. Biochem. Pharmacol. 63, 41-47.

Lanzotti, V., 2006. The analysis of onion and garlic. J. Chromatogr. A 1112, 3-22.

Le Bon, A.-M., Roy, C., Dupont, C., Suschetet, M., 1997. In vivo antigenotoxic effects of dietary allyl sulfides in the rat. Cancer Lett. 114, 131-134.

Lee, K.-S., Lee, B.-S., Semnani, S., Avanesian, A., Um, C.-Y., Jeon, H.-J., Seong, K.-M., Yu, K., Min, K.-J., Jafari, M., 2010. Curcumin extends life span, improves health span, and modulates the expression of age-associated aging genes in Drosophila melanogaster. Rejuv. Res. 13, 561-570.

Lei, M., Xu, M., Zhang, Z., Zhang, M., Gao, Y., 2014. The analysis of saccharide in black garlic and its antioxidant activity. Adv. J. Food Sci. Technol. 6, 755-760.

Lin, Y.-T., Yang, J.-S., Lin, S.-Y., Tan, T.-W., Ho, C.-C., Hsia, T.-C., Chiu, T.-H., Yu, C.-S., Lu, H.-F., Weng, Y.-S., 2008. Diallyl disulfide (DADS) induces apoptosis in human cervical cancer Ca Ski cells via reactive oxygen species and Ca2+-dependent mitochondria-dependent pathway. Anticancer Res. 28, 2791-2799.

Lu, H., Sue, C., Yu, C., Chen, S., Chen, G., Chung, J., 2004. Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. Food Chem. Toxicol. 42, 1543-1552.

Martínez, A., Ikken, Y., Cambero, M.I., Marín, M.L., Haza, A.I., Casas, C., Morales, P., 1999. Mutagenicity and cytotoxicity of fruits and vegetables evaluated by the Ames test and 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay / Mutagenicidad y citotoxicidad de frutas y vegetales evaluadas por el test de Ames y el ensayo del bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT). Food Sci. Technol. Int. 5, 431-437.

Masuda, Y., Asada, K., Satoh, R., Takada, K., Kitajima, J., 2015. Capillin, a major constituent of Artemisia capillaris Thunb. flower essential oil, induces apoptosis through the mitochondrial pathway in human leukemia HL-60 cells. Phytomedicine 22, 545-552.

Merhi, F., Auger, J., Rendu, F., Bauvois, B., 2008. Allium compounds, dipropyl and dimethyl thiosulfinates as antiproliferative and differentiating agents of human acute myeloid leukemia cell lines. Biologics 2, 885.

Mukhopadhyay, I., Chowdhuri, D.K., Bajpayee, M., Dhawan, A., 2004. Evaluation of in vivo genotoxicity of cypermethrin in Drosophila melanogaster using the alkaline Comet assay. Mutagenesis 19, 85-90.

Musk, S., Clapham, P., Johnson, I., 1997. Cytotoxicity and genotoxicity of diallyl sulfide and diallyl disulfide towards Chinese hamster ovary cells. Food Chem. Toxicol. 35, 379-385.

Olive, P.L., Banáth, J.P., 2006. The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1, 23.

Park, J.-H., Park, Y.K., Park, E., 2009. Antioxidative and antigenotoxic effects of garlic (Allium sativum L.) prepared by different processing methods. Plant Foods Hum. Nutr. 64, 244-249.

Penner, R., Fedorak, R.N., Madsen, K.L., 2005. Probiotics and nutraceuticals: non-medicinal treatments of gastrointestinal diseases. Curr. Opin. Pharmacol. 5, 596-603.

Prakash, D., Singh, B.N., Upadhyay, G., 2007. Antioxidant and free radical scavenging activities of phenols from onion (Allium cepa). Food Chem. 102, 1389-1393.

Prowse, G.M., Galloway, T.S., Foggo, A., 2006. Insecticidal activity of garlic juice in two dipteran pests. Agr. Forest Entomol. 8, 1-6.

Ramel, C., Alekperov, U., Ames, B., Kada, T., Wattenberg, L., 1986. Inhibitors of mutagenesis and their relevance to carcinogenesis: report by ICPEMC expert group on antimutagens and desmutagens. Mutat. Res. 168, 47-65.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., Bier, E., 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res. 11, 1114-1125.

Ren, N., Charlton, J., Adler, P.N., 2007. The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. Genetics 176, 2223-2234.

Rivlin, R.S., 2001. Historical perspective on the use of garlic. J. Nutr. 131, 951S-954S.

Romero-Jimenez, M., Campos-Sanchez, J., Analla, M., Muñoz-Serrano, A., Alonso-Moraga, Á., 2005. Genotoxicity and anti-genotoxicity of some traditional medicinal herbs. Mutat. Res. 585, 147-155.

Seki, T., Tsuji, K., Hayato, Y., Moritomo, T., Ariga, T., 2000. Garlic and onion oils inhibit proliferation and induce differentiation of HL-60 cells. Cancer Lett. 160, 29-35.

Sengupta, A., Ghosh, S., Bhattacharjee, S., 2004. Allium vegetables in cancer prevention: an overview. Asian Pac. J. Cancer P. 5, 237-245.

Sengupta, A., Ghosh, S., Das, S., 2002. Administration of garlic and tomato can protect from carcinogen induced clastogenicity. Nutr. Res. 22, 859-866.

Serpeloni, J.M., dos Reis, M.B., Rodrigues, J., dos Santos, L.C., Vilegas, W., Varanda, E.A., Dokkedal, A.L., Cólus, I.M.S., 2008. In vivo assessment of DNA damage and protective effects of extracts from Miconia species using the comet assay and micronucleus test. Mutagenesis 23, 501-507.

Shaath, N.A., Flores, F.B., 1998. Egyptian onion oil. Dev. Food Sci. 40, 443-453.

Shrivastava, S., Ganesh, N., 2010. Tumor inhibition and Cytotoxicity assay by aqueous extract of onion (Allium cepa) & Garlic (Allium sativum): an in-vitro analysis. Int. J. Phytomed. 2, 80-84.

Shukla, Y., Kalra, N., 2007. Cancer chemoprevention with garlic and its constituents. Cancer Lett. 247, 167-181.

Shukla, Y., Taneja, P., 2002. Antimutagenic effects of garlic extract on chromosomal aberrations. Cancer Lett. 176, 31-36.

Siddique, H.R., Chowdhuri, D.K., Saxena, D.K., Dhawan, A., 2005. Validation of Drosophila melanogaster as an in vivo model for genotoxicity assessment using modified alkaline Comet assay. Mutagenesis 20, 285-290.

Singh, S., Abraham, S., Kesavan, P., 1996. Radioprotection of mice following garlic pretreatment. Brit. J. Cancer. 27, S102.

Singh, S.V., Pan, S.S., Srivastava, S.K., Xia, H., Hu, X., Zaren, H.A., Orchard, J.L., 1998. Differential induction of NAD (P) H: quinone oxidoreductase by anti-carcinogenic organosulfides from garlic. Biochem. Bioph. Res. Co. 244, 917-920.

Sowjanya, B.L., Devi, K.R., Madhavi, D., 2009. Modulatory effects of garlic extract against the cyclophosphamide induced genotoxicity in human lymphocytes in vitro.

Sundaram, S.G., Milner, J.A., 1996a. Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis 17, 669-673.

Sundaram, S.G., Milner, J.A., 1996b. Diallyl disulfide inhibits the proliferation of human tumor cells in culture. BBA-Mol. Basis Dis. 1315, 15-20.

Tan, H., Ling, H., He, J., Yi, L., Zhou, J., Lin, M., Su, Q., 2008. Inhibition of ERK and activation of p38 are involved in diallyl disulfide induced apoptosis of leukemia HL-60 cells. Arch. Pharm. Res. 31, 786-793.

Tanaka, S., Haruma, K., Yoshihara, M., Kajiyama, G., Kira, K., Amagase, H., Chayama, K., 2006. Aged garlic extract has potential suppressive effect on colorectal adenomas in humans. J. Nutr. 136, 821S-826S.

Tasset-Cuevas, I., Fernández-Bedmar, Z., Lozano-Baena, M.D., Campos-Sánchez, J., de Haro-Bailón, A., Muñoz-Serrano, A., Alonso-Moraga, Á., 2013. Protective Effect of Borage Seed Oil and Gamma Linolenic Acid on DNA: In Vivo and In Vitro Studies. PLoS ONE 8.

Trichopoulou, A., Vasilopoulou, E., 2000. Mediterranean diet and longevity. Brit. J. Nutr. 84, S205-S209.

Wang, Y., Tian, W.-X., Ma, X.-F., 2012. Inhibitory effects of onion (Allium cepa L.) extract on proliferation of cancer cells and adipocytes via inhibiting fatty acid synthase. Asian Pac. J. Cancer P. 13, 5573-5579.

Wu, X.-J., Kassie, F., Mersch-Sundermann, V., 2005. The role of reactive oxygen species (ROS) production on diallyl disulfide (DADS) induced apoptosis and cell cycle arrest in human A549 lung carcinoma cells. Mutat. Res.- Fund. Mol. M. 579, 115-124.

Wu, X.-j., Stahl, T., Hu, Y., Kassie, F., Mersch-Sundermann, V., 2006. The production of reactive oxygen species and the mitochondrial membrane potential are modulated during onion oil–induced cell cycle arrest and apoptosis in A549 cells. J. Nutr. 136, 608-613.

Yamamoto, Y., Aoyama, S., Hamaguchi, N., Rhi, G.-S., 2005. Antioxidative and antihypertensive effects of Welsh onion on rats fed with a high-fat high-sucrose diet. Biosci. Biotech. Bioch. 69, 1311-1317.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., Adler, P.N., 2008. The multiple-wing-hairs gene encodes a novel GBD–FH3 domain-containing protein that functions both prior to and after wing hair initiation. Genetics 180, 219-228.

Yang, J.-S., Chen, G.-W., Hsia, T.-C., Ho, H.-C., Ho, C.-C., Lin, M.-W., Lin, S.-S., Yeh, R.-D., Ip, S.-W., Lu, H.-F., 2009. Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO 205) through the induction of reactive oxygen species, endoplasmic reticulum stress, caspases casade and mitochondrial-dependent pathways. Food Chem. Toxicol. 47, 171-179.

Yang, J., Meyers, K.J., van der Heide, J., Liu, R.H., 2004. Varietal differences in phenolic content and antioxidant and antiproliferative activities of onions. J. Agr. Food Chem. 52, 6787-6793.

Yoshinari, O., Shiojima, Y., Igarashi, K., 2012. Anti-obesity effects of onion extract in Zucker diabetic fatty rats. Nutrients 4, 1518-1526.

Zhang, Z., Han, S., Wang, H., Wang, T., 2014. Lutein extends the lifespan of Drosophila melanogaster. Arch. Gerontol. Geriat. 58, 153-159.

<u>CAPÍTULO IV</u>: New Targets on the Biological Activities of Lycopene and Tomato.

Chapter IV

Artículo en preparación

ABSTRACT

Tomato constitutes a worldwide important crop and an essential part of the now a day's human diet. There are many researches based on the use of tomato for prevention at cardiovascular level. Nevertheless tomato anticancer studies are scarce. The aim of the present study was to evaluate the nutraceutical potential of tomato as well as lycopene and to consider a new nutraceutical value for this fruit regarding to the protection against genetic damage and as chemo-preventive agent. To this purpose, we assessed on the genotoxicity, anti-genotoxicity and lifespan properties of this vegetable and its representative carotenoid using a *Drosophila in vivo* model (wing spot test) and the cytotoxic and pro-apoptotic activity (internucleosomal DNA fragmentation and DNA strand break) using HL60 *in vitro* model. Results showed that i) Tomato and lycopene are safe and antimutagenic against H_2O_2-induced damage; ii) Respect to the lifespan, tomato and lycopene are harmless at the lowest concentration. In general, both lifespan and healthspan reductions by lycopene are highest than tomato; iii) Tomato is cytotoxic in a dose-dependent manner, lycopene although it is not; iv) Tomato and lycopene do not induce DNA-fragmentation but they induce significant clastogenic activity at low level in the leukaemia cells.

Based on the protection of the oxidative DNA damage and the possible chemo-preventive activity, tomato is the best candidate to be considered as nutraceutical substance. Furthermore, synergistic action of other components within tomato matrix could be the cause of the health effects observed in this vegetable, which are not fully explained by lycopene.

Key words: tomato, lycopene, antigenotoxicity, lifespan, clastogenic activity

INTRODUCTION

The role of the diet and nutrition as determinants of chronic diseases is well recognized (Who and Consultation, 2003).Palaeolithic diet is an ancestral diet which its principal components are lean meat, fish, vegetables, fruits, roots, eggs and nuts and processed foods as olive oil are excluded (Klonoff, 2009). This ancestral diet has higher antioxidant content than current diets (Benzie, 2003) being considered as a good model to prevent age-related degenerative diseases (Lindeberg et al., 2003). For this reason, Mediterranean diet due to its similar components respect to this ancestral diet is an extend focus of researches.

Worldwide, tomato constitutes an important crop and an integral part of the now a day's human diet. Its worldwide production for 2013 was estimated in 163,963,770 tones according FAO (2015). Furthermore, according to the European Commission (2014), tomato was the first vegetable crop in Europe with an estimated production of 14.9 million tonnes in 2013, of which approximately two thirds came from Italy and Spain. The wild relatives of the cultivated tomatoes descent from America and were introduced in Europe on the sixteenth century (1540 in Spain) (Peralta and Spooner, 2006). This vegetable contains many bioactive molecules, including those that act as antioxidants, such as ascorbic acid, vitamin E, carotenoids, flavonoids and phenolic acid (Toor and Savage, 2005). Tomato fruit has assumed the status of functional food due to the association between its consumption and a reduced likelihood of certain

types of cancers and cardiovascular diseases (Canene-Adams et al., 2005; Palozza et al., 2009; Silaste et al., 2007; Willcox et al., 2003). This vegetable is not genotoxic in *Drosophila melanogaster* (Dutra et al., 2009), reduces the mutagenicity induced by aflatoxin B1 (AFB1), benzo[a] pyreno (BaP), cyclophosphamide (CP) and 2-amino-3-methylimidazo [4,5-f] quinolone (IQ) in *S. typhimurium (Rauscher et al., 1998; Rodríguez-Muñoz et al., 2009)*, protects against 7,12 dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG)-induced genetic damage in mice (Bhuvaneswari et al., 2004; Velmurugan et al., 2004a), has an anticarcinogenic effect in Swiss albino and C57 Bl mice (Agrawal et al., 2009) and is cytotoxic in different cancer cell lines (Friedman et al., 2009)

Lycopene is one of over 600 carotenoids found in nature. The richest source of lycopene in the diet is tomato and tomato derived products. Lycopene content ranged in fresh tomato from 0.85 mg/100 g to 13.6 mg/100g (Lugasi et al., 2003). This molecule is the most predominant carotenoid in human plasma but its level is affected by several biological and lifestyle factors. Lycopene decreases the risk of chronic diseases such as cancer and cardiovascular diseases (Agarwal and Rao, 2000). It is a potent antioxidant and has a singlet-oxygen-quenching ability twice as high as that β-carotene (Di Mascio et al., 1989). Lycopene is not genotoxic (Velmurugan et al., 2004b); modulates the toxicity and genotoxicity of different genotoxines, such as H_2O_2, n-nitrosodiethylamine (DEN) and AFB1 (Reddy et al., 2006; Scolastici et al., 2008); increases the longevity (DAI et al., 2008; Hu et al., 2013); inhibits the cell growth and induces apoptosis of different malignant cell lines (Hantz et al., 2005; Salman et al., 2007).

The aim of the present study was to evaluate the nutraceutical potential of tomato as well as lycopene, the main carotenoid presents in this complex matrix and to consider a new nutraceutical value for this fruit regarding to the protection against genetic damage and as chemo-preventive agent. This is the first step in the research of preventive processes. To this purpose, we determinate the genotoxicity, anti-genotoxicity and lifespan properties of this vegetable and its representative carotenoid using *Drosophila in vivo* model and the cytotoxic and apoptotic effect using HL60 *in vitro* model.

MATERIAL AND METHODS

Preparation of selected samples

Tomato fruits were purchased in a local market, thoroughly washed with tap water and rinsed with distilled water, cut in slim slices and freeze-dried at -80ºC for 3 days. After that, the tomato samples were lyophilized, pulverized with a mortar pestle, sieved and stored at 25ºC in dark until use. Lycopene, the selected single compound, was purchased from Sigma (Cat. Number 068K5166), solved in ethanol and filtered before use.

No official recommendations for tomato intake were found. For this reason, the tomato concentration range was calculated considering that tomato is one component of the total intake vegetable (approximately 10%). The tomato concentrations were established taking into account the average daily food intake of *Drosophila melanogaster* (1mg/mL) and the average body weight of this genetic animal model (1 mg).

In vivo assays

Drosophila melanogaster strains

In vivo studies (genotoxicity, antigenotoxicity and longevity) were carried out in *Drosophila melanogaster,* a widely employed genetic animal model due to its homology with mammal models in biological, physiological and neurological properties, being an ideal model to the evaluation of complex mixtures and foods. Furthermore, about 75 % of human disease-causing genes are believed to have a functional homolog in the fly (Jones and Grotewiel, 2011; Reiter et al., 2001). In addition, *Drosophila melanogaster* has a short lifespan compared with similar *in vivo* models, reducing the experimental periods and finally it has similar human metabolic pathways controlling nutrient uptake, storage and metabolism (Anh et al., 2011; Zhang et al., 2014). For these reasons *D. melanogaster* is a good model that can help to understand aging in other animals including human.

Fly strains and maintenance

Two fly strains carrying visible hair markers in the third chromosome were used in our experimental design:

- *mwh/mwh* (multiple wing hair strain), mwh marker (*mwh, 3_0.3*) is a recessive mutation which produces multiple tricomas per cell instead of one (Yan et al., 2008).
- *flr^3/In (3LR) TM3, rip^p sep bx^{34e} e^s Bd^s* (flare strain), flare marker (*flr^3, 3_38.3*) is a homozygous recessive lethal mutation which produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al., 2007).

Both strains were maintained at 25ºC and 80% humidity in glass tubes with homemade meal (0.5 g NaCl, 12 g agar-agar, 100 g yeast, 25 g sucrose, 5 mL propionic acid, 3.5 mL of a 0.2% sulphate streptomycin solution and 1 L distilled water) making changes three times per week.

Virgin females were obtained from these tubes and then were crossed in order to perform the crosses for the different assays.

Genotoxicity and Antigenotoxicity assays

Genotoxicity assays were carried out following the method described by Graf et al. (1984) using pure water as negative control. Briefly, 200 optimally virgin females (4 days old) of flr^3 strain were crossed with 100 males of *mwh* strain during 8 hours. After 72 h, F1 transheterozygous larvae were collected, washed with distilled water and placed in the treatment tubes where they were fed chronically with the different tested samples. The treatment tubes contained 0.85 g of *Drosophila* Instant Medium and 4 mL of solutions with different tested concentrations of the sample of interest (0.625, 1.25, 2.5 and 5 mg/mL for tomato and 7, 14, 28 and 56 µM for Lycopene).

The antigenotoxicity assays were similar to genotoxicity assay but all the treatment used in genotoxicity were concurrently supplemented with H_2O_2 (0.12 M) as positive genotoxicant control. The emerged adults in each group were finally stored in 70 % ethanol until the wings were mounted to the scrutiny of the mutations.

Mutation scoring

Wings of trans-heterozygous individuals (*mwh* flr^+/mwh^+ flr^3) of each control and concentration were mounted on slides with Faure´s solution. Both ventral and dorsal surfaces of the wings containing 22,000 cells were inspected under a photonic microscope at 400x magnification. Similar number of male and female wings was mounted. On marker-heterozygous wings, two types of spots were observed: (i) single spots (*mwh* and flr^3), which are produced by somatic mutation, chromosome aberration as well as mitotic recombination, and (ii) twin spots (*mwh* and flr^3 in the same clone), which are produced exclusively from mitotic recombination.

Data evaluation and statistical analysis

Three type of spots were classified: small single spots (1-2 cells), large single spots (>2 cells) and twin spots (Graf et al., 1984). Evaluation of the genotoxicity of tomato and lycopene was based on the comparison of frequencies of the total spots per wing between treatment group and the negative control. The statistical significance of the results was determined with a multiple-decision procedure and was applied to determine whether a result is positive, inconclusive or negative (Graf et al., 1998). Inconclusive and positive results were analyzed with the non-parametric U-test of Mann Whitney and Wilcoxon, with significance levels $\alpha = \beta = 0.05$. The inhibition percentage (IP) were calculated from total spots per wing with the following formula (Abraham, 1994):

IP = [(*single genotoxine – combined treatment/ single genotoxine*] x 100

Lifespan and healthspan assays

In order to obtain comparable results, we selected animals which exhibited the same genotype as in the genotoxicity assays. The F1 individuals obtained from mwh and flr^3 parental strains by egg laying of 24 h in yeast was used. These assays were carried out at 25ºC. Briefly, synchronized trans-heterozygous 72±12 h larvae were washed and transferred into groups of 100 individuals in vials with a mixture of 0.85 g of *Drosophila* Instant Medium and 4 mL of different concentrations of the two selected substances. Emerged adults were collected under CO_2 anesthesia and placed in groups of 10 individuals of the same sex into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 mL of different concentrations of the tested compounds. Three replicates were followed during the complete life extension for each control and established concentrations. The survival number was counted and media renewed twice a week.

Statistical analysis

The statistical treatment of survival data for each concentration and respective control was assessed using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA), applying the Kaplan-Meier method. The survival function for each treatment is plotted as survival curves. The significance of these was determined using the Log-Rank method (Mantel-Cox).

In vitro assays

Cells and cell culture conditions

Human promyelotic leukaemia cell line (HL60) was provided by Dr. Villalba (Department Cell Biology, University of Córdoba, Spain). HL60 cells were incubated in RPMI-1640 medium (Sigma, R5886) supplemented with L-glutamine 200 mM, (Sigma, G7513), antibiotic-antimycotic solution (Sigma, A5955) and 10% heat-inactivated foetal bovine serum (Linus, S01805). Cell cultures were plated at a 2.5x 10^5 cells/mL density and passed every 2-3 days.

Cell growth inhibition study (cytotoxic activity)

Cell growth inhibition was carried out using the Trypan Blue dye exclusion test (Sigma, T8154). HL60 cells were placed in 96 well culture plates (1x10^5 cell/mL) and cultured for 72 h supplemented with a wide range of tested concentrations (0.125-4 mg/mL) for tomato and 1.2-44.8 µM for lycopene) in CO_2 incubator. After incubation period, cells were stained with Trypan Blue dye (1:1 v/v) and counted under inverted microscope using a Neubauer chamber at 100X magnification. Cell viability curves are expressed as the survival percentage of each treatment compared with the control. IC_{50} values (the cytotoxic inhibitory concentration 50) were established.

DNA fragmentation assays.

HL60 cells (1.5x10^6 cells/mL) were treated with supplemented medium containing different concentrations of tomato and lycopene (as selected in the cytotoxic assay) during 5 h. After treatment period, cells were washed

with PBS. Genomic DNA was extracted using a commercial DNA-extraction kit (Blood Genomic DNA Extraction Mini Spin Kit, Canvax Biotech, Cordoba, Spain). Subsequently, a RNase treatment on DNA was carried out overnight at 37ºC. Finally, 1500 ng DNA per sample were loaded, electrophoresed in a 2% agarose gel for 120 min at 60 V and stained with ethidium bromide. Internucleosomal DNA fragmentation was determined by the presence of a ladder band patterns with 200 bp multiple fragments under UV light.

Comet assay: DNA damage evaluation

The ability of the compounds to produce strand breaks in the DNA structure was assessed by the alkaline comet assay according to Olive and Banáth (2006) with slight modifications. HL60 cells ($5x10^5$ cells) were plated in 1.5ml of culture medium supplemented with different concentrations of tomato (0.125, 0.25 and 0.5 mg/mL) and lycopene (1.2, 2.4 and 4.8 µM) and incubated for 5 h. After treatment, cells were washed and adjusted to 6.25×10^5 cells/ml in PBS. Then, cells (1.6×10^4) were suspended in 75 µL prewarmed low melting point agarose (A4018, Sigma) and 50 µL of the suspension were rapidly spread on microscope slides and covered with coverslips. After gelling for 30 min at RT, the coverslips was gently removed and the slides were put in a tank filled with lysis solution (2.5M NaCl, 100mM Na-EDTA, 10mM Tris, 250mM NaOH, 10% DMSO and 1% Triton X-100; pH= 13) for 1 h at 4ºC. The slides were then removed from the lysis solution and incubated in alkaline electrophoresis buffer (300mM NaOH and 1mM Na-EDTA, pH= 13) for 20-30 min at 4ºC. Electrophoresis was then carried out in a fresh electrophoresis buffer for 15 minutes at 20 V and 400 mA in dark conditions. After electrophoresis, slices were gently washed in

cold fresh neutralization buffer (0.4M Tris-HCl buffer, pH 7.5) for 10 min. After drying overnight at RT in dark conditions, gels were stained with 7 µL propidium iodide and covered with a coverslip. Finally, the slides were dried overnight at RT in dark conditions. Gels were then photographed in at X400 magnification in a Leica DM2500 microscope. At least 50 cells were selected to each treatment and were analysed using the Open Comet™ software (Gyori et al., 2014).The statistical ANOVA-Tukey test was applied (Serpeloni et al., 2008) using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA) in order to compare the results obtained for the different treatments with the negative control

RESULTS

In vivo assays

Genotoxic/Antigenotoxic activity of the tested compounds

Table 1 shows the results obtained with tomato and lycopene in the wing-spot test using *Drosophila* standard cross. Negative control (distilled water) showed a frequency of mutations per wings of 0.225 which fall into the historical range for wing spot test (Fernández-Bedmar et al., 2011; Rojas-Molina et al., 2005). The mutation frequencies were higher at both tested concentrations than the negative control but no significant results (non-genotoxic) were yielded in both compounds at all tested concentrations by the Mann Whitney U-test. A dose effect was observed in the number of total spots per wing.

Table 1 also shows the results of antigenotoxicity. Hydrogen peroxide is a well-known genotoxin. This molecule is able to induce somatic mutation

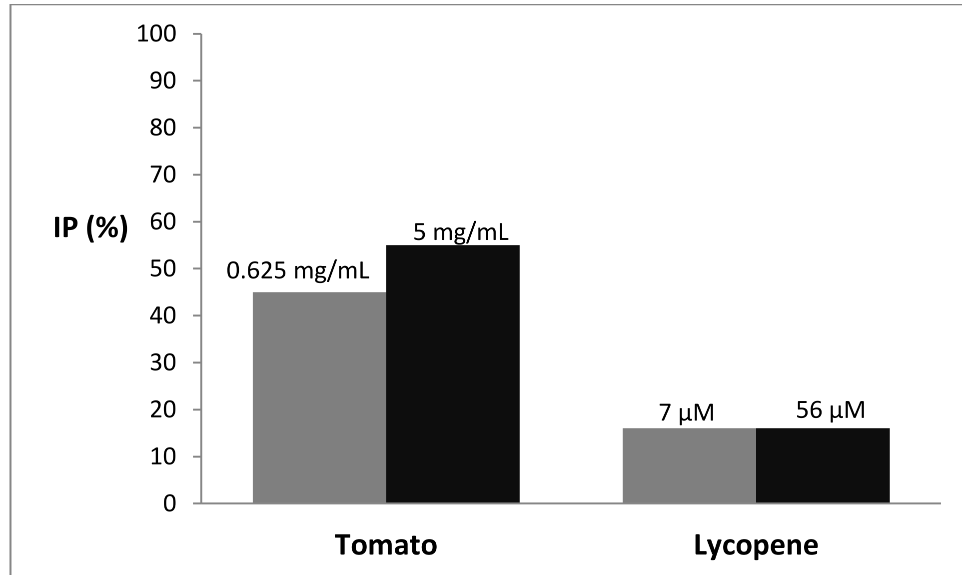
and mitotic recombination in *Drosophila melanogaster* (Romero-Jimenez et al., 2005). H_2O_2 resulted mutagenic as expected (0.5 total spots per wing) falling this mutation rate into the historical normal range published for the genotoxic activity of this oxidative molecule (Fernández-Bedmar et al., 2011). Co-treatments with tomato and H_2O_2 simultaneously showed a dose-

Table1. Genotoxicity and antigenotoxicity results of tomato and lycopene obtained in the *Drosophila* wing spot test.

Compounds	N	Small spots (1-2 cells)	Large spots (>2 cells)	Twin spots	Total spots
Controls					
H_2O	40	0.15 (6)[a]	0.075 (3)	0 (0)	0.225 (9)
H_2O_2(0.12M)	40	0.25 (10)	0.25 (10)	0 (0)	0.5 (20)*
Tomato (mg/mL)					
0.625	40	0.225 (9)	0.05 (2)	0 (0)	0.275 (11)ns
5	40	0.25 (10)	0.125 (5)	0 (0)	0.375 (15)ns
0.625 + H_2O_2	40	0.25 (10)	0 (0)	0.025 (1)	0.275 (11)ns
5 + H_2O_2	40	0.225 (9)	0 (0)	0 (0)	0.225 (9)ns
Lycopene (µM)					
7	40	0.175 (7)	0.025 (1)	0 (0)	0.2 (8)ns
56	40	0.35 (14)	0.025 (1)	0 (0)	0.375 (15)ns
7 + H_2O_2	40	0.42 (17)	0 (0)	0 (0)	0.42 (17)ns
56 + H_2O_2	40	0.3 (12)	0.05 (2)	0.075 (3)	0.42 (17)ns

N: number of wings; a: number of spots per wing *: significant (P≤ 0.05); The data were evaluated by the non-parametric U-test of Mann, Whitney and Wilcoxon (Frei and Würgler, 1995)

Figure 1. Inhibition effects of tomato and lycopene against H_2O_2-induced genetic damage.



dependent inhibitory effect against H_2O_2-genetic damage, reaching 55 % of inhibition at the highest tested concentration (5 mg/mL). Lycopene reduced the H_2O_2-genotoxic effect in the same way at the two tested concentrations (7 and 56 µM); the inhibition percentage in both concentrations was 16 % (**Figure 1**).

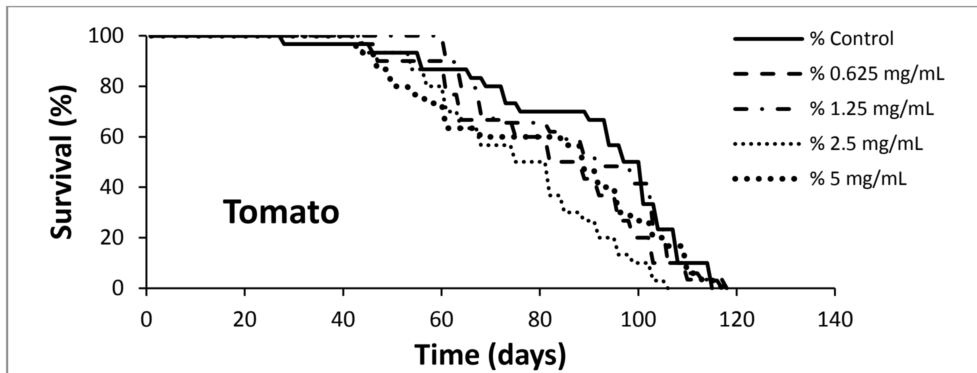
Effect of tomato and lycopene on longevity and healthspan

Tomato and lycopene showed a general negative effect on lifespan of *Drosophila melanogaster*. The dose-effect relationship is illustrated in **Figure 2**. As shown in **Table 2**, the control group had in both treatments (tomato and lycopene) a mean lifespan of 90.1 days. Nevertheless, the different treatments decreased the mean lifespan at all tested concentration. The Kaplan-Meier test demonstrated that only

supplementation with 2.5 mg tomato/mL diet significantly decreased the mean lifespan by 16%. Lycopene had the same effect on Drosophila lifespan, decreasing significantly the mean lifespan at 14 and 56 µM by 20% and 16% respectively.

Figure 2. Survivalcurves of *D. melanogaster* fed with different concentrations of tomato (A) and lycopene (B).

(A)



(B)

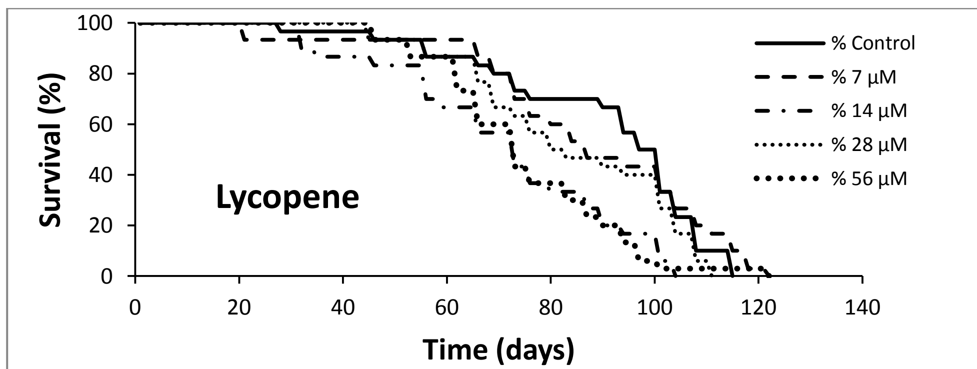


Table 2. Effect of different tomato and lycopene treatments on the survival time of *Drosophila melanogaster*.

	Mean lifespan (days)	Mean lifespan difference (%)[a]	Healthspan (75th percentile) (days)	Healthspan difference (%)[a]
Tomato (mg/mL)				
Control	90.1±4.07		58.4±5.48	
0.625	82.2±3.79	-9	55.7±2.90	-5
1.25	88.4±3.49	-2	63.5±0.94	9
2.5	76.0±3.30***	-16	54.0±2.37	-7
5	81.2±4.47	-10	49.1±1.63*	-16
Lycopene (µM)				
Control	90.1±4.07		58.4±5.48	
7	88.2±4.74	-2	57.2±7.97	-2
14	72.3±4.11***	-20	43.1±4.10*	-26
28	84.1±3.72	-7	58.9±3.28	0
56	75.4±3.19**	-16	55.7±2.54	-5

[a] The difference was calculated by comparing treated flies to the concurrent water control. Positive numbers indicate lifespan increase and negative numbers indicate lifespan decrease. *p≤0.05, **p≤0.01, ***p≤0.001significances obtained by using the log rank (Mantel-Cox) tests.

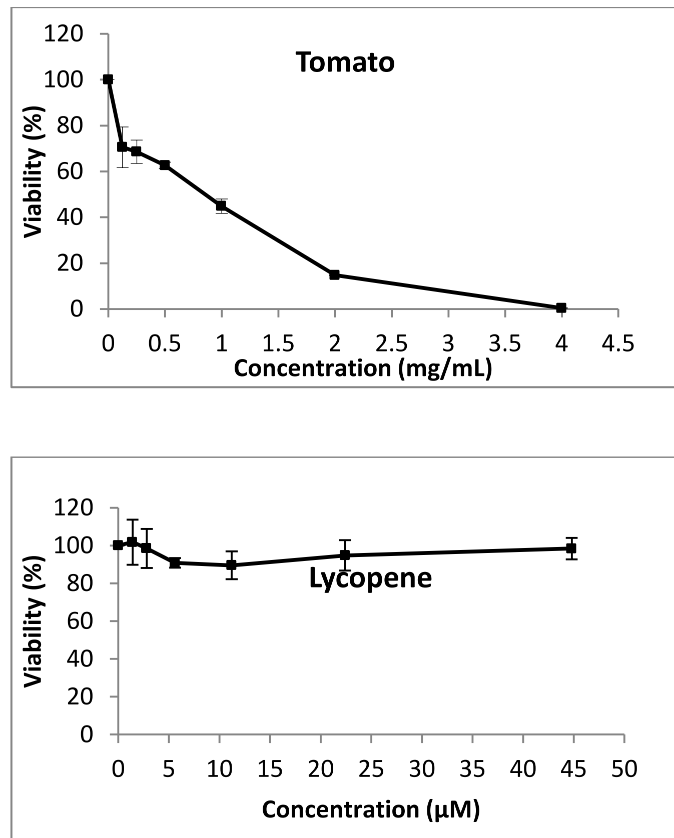
Table 2 also shows healthspan results (portion ≥ 75% of lifespan curves). All tomato concentrations decreased the average healthspan of flies. Nevertheless, only supplementation with 5 mg tomato/mL diet significantly decreased healthspan by 16% compared with the control group. In the same way, lycopene also significantly decreased healthspan at 14 µM by 26%.

In vitro assays

<u>Cytotoxicity assay</u>

Tomato was able to inhibit the cell growth of the HL60 cell line with a dose-response effect. Nevertheless, lycopene did not induce any visible cytotoxic effect on HL60 tumour cells. The IC_{50} of tomato was 0.8 mg/mL (**Figure 3**).

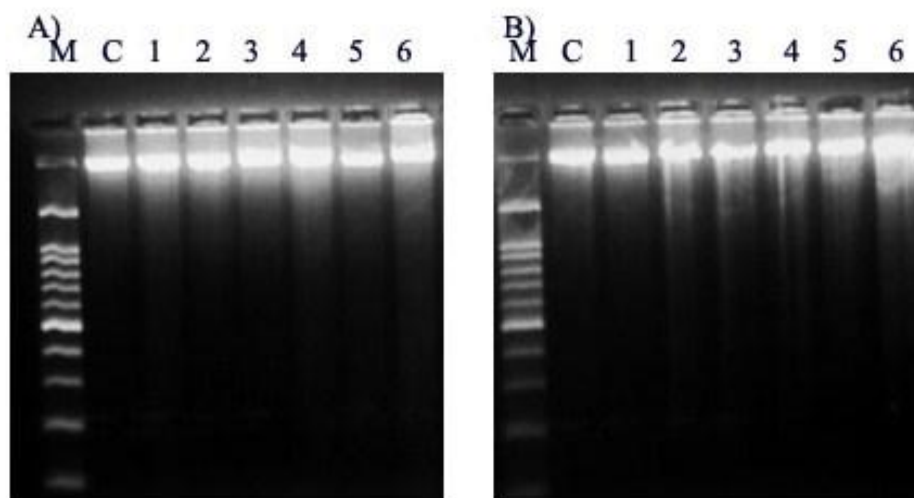
Figure 3. Cytotoxic effects on HL60 cells at 72 h of treatment.



DNA integrity.

Apoptosis is a major mechanism of cancer suppression. In an attempt to determine the mechanism of the cytotoxic effect on HL60 cell line, we examined if our compounds could induce programmed cell death. The results are shown in **Figure 4**. DNA fragmentation into nucleosomal 180-200 bp units was not found in the human promyelocytic leukaemia cells when the media was supplemented with different concentrations of tomato and lycopene (0.125-4 mg/mL and 1.4-44.8 µM respectively).

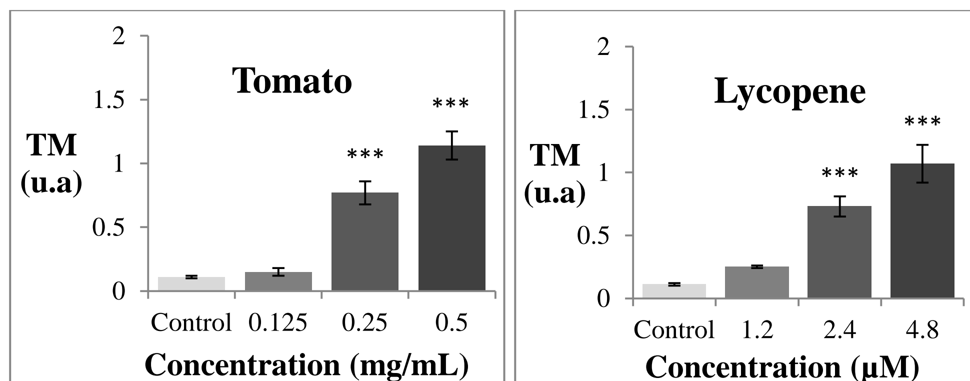
Figure 4. DNA fragmentation induced in HL-60 cells by tomato and lycopene. HL60 human leukemia cells were exposed for 5 hours to different concentrations of test treatments. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min.



Tomato (A): Control (lane C), 0.125 mg/mL (lane 1), 0.25 mg/mL (lane 2), 0.5 mg/mL (lane 3), 1 mg/mL (lane 4), 2 mg/mL (lane 5), 4 mg/mL (lane 6). **Lycopene (B)**: Control (lane C), 1.4 µM (lane 1), 2.8 µM (lane 2), 5.6 µM (lane 3), 11.2 µM (lane 4), 22.4 µM (lane 5), 44.8 µM (lane 6).

In order to determine the capacity of tomato and lycopene to induce DNA single strand breaks was used the alkaline comet assay. Cytotoxic and DNA internucleosomal fragmentation results were necessary to decide the tested concentrations of each compound. As illustrated **Figure 5**, all concentrations of tomato (0.125, 0.25 and 0.5 mg/mL) and lycopene (1.2, 2.4 and 4.8 µM) increased the tail moment (TM) parameter with a dose-response effect. Nevertheless, only the highest tested concentrations of tomato and lycopene significantly increased the TM parameter.

Figure 5. Alkaline single cell gel electrophoresis (pH<13) of promielocitic human leukaemia cell line (HL60) after 5h-treatment with different concentrations of tomato(A) and lycopene (B). DNA migration is reported as mean TM. The plot shows mean TM values, standard errors and significance compared to the non-treated control, p ≤ 0.001 (***).



DISCUSSION

Nowadays there are many researches based on the use of tomato for prevention at cardiovascular level. Nevertheless tomato anticancer studies are scarce. To the best of our knowledge, it is the first multidisciplinary and

integrative study on the nutraceutical value and safety of tomato and lycopene with respect to their biological activities at three individual, cell and DNA levels.

The DNA stability or lack of genotoxicity observed in all concentrations of tomato and lycopene confirm their safety to be consumed. These results agree with those obtained by Dutra et al. (2009) with aqueous organic tomato extract at higher concentrations than ours (100%= 1g/ mL) in the same *in vivo* genetic model. Studies with mice have also demonstrated the safe use of tomato paste and tomato juice using micronucleus test (ASITA ET AL., 2008; VELMURUGAN ET AL., 2004A). In addition, tomato oleoresins were not mutagenic to bacteria at 200, 300 and 400 µM for the TA 100 tester strain of *S. typhimurium* (Rodríguez-Muñoz et al., 2009). It is the first time that the safety of lycopene using Drosophila melanogaster *in vivo* model is demonstrated. Lycopene was not found to induce mutations at the *tk* locus in mouse lymphoma cells. Neither this carotenoids induced clastogenic or spindle damaging effect in mouse bone marrow (McClain and Bausch, 2003).

Studies on molecules or complex mixtures that modulate carcinogen-induced genotoxic effects in animal models are used to assess the antimutagenic or anticarcinogenic properties of putative chemopreventive compounds. We determined that all assayed concentrations of tomato and lycopene exerted an antignotoxic effect against H_2O_2-induced damage in *Drosophila* model. Different tomato extracts (n-hexane, dichloremethane, acetone and 2-propanol) had antimutagenic effect against aflatoxin B_1, benzo [a] pyrene, cyclophosphamide and 2-amino-3-methylimidazo 4,5- w

x f quinolone-induced DNA damage in *S. typhimurium* using the Ames test (Rauscher et al., 1998). Furthermore Bhuvaneswari et al. (2004) yield positive antigenotoxic results of tomato (500 mg/kg body weight) against 7,12-dimethylbenz[a]anthracene-induced genotoxicity in mice. Nevertheless Asita et al. (2008) observed that the tomato juice did not have an inhibitory effects against cyclophosphamide-induced genotoxicity at different concentrations (0.25, 0.5 and 1 g/mL) in mice. This discrepancy can be due to the different genetic background and methods used.

Lycopene is known as the most effective antioxidant among the carotenoids. We agree with the results obtained by other authors. This molecule significantly reduced the n-nitrosodiethylamine- and H_2O_2-induced genotoxicity at 10, 25 and 50 µM (Scolastici et al., 2008). Pre-treatments with lycopene (1.25 mg/Kg) using Wistar rats, significantly reduced the frequency of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced bone marrow micronuclei and chromosomal aberrations (Velmurugan et al., 2004b)

Also, it is interesting that the antigenotoxic effect of tomato is higher than lycopene. This can be due to tomato is a reservoir of diverse molecules with an antioxidant activity, such us ascorbic acid, vitamin E, flavonoids, phenolic acids and other carotenoids (George et al., 2004; Verhoeyen et al., 2002). These molecules could be acting synergistly.

Drosophila melanogaster has been used as a model in aging research for decades. Flies are well suited for such studies due to a number of reasons, as they share many conserved biological pathways and also because of

their relative short life span, tiny body size and easy maintenance (Minois, 2006). To our knowledge, it is the first time that is studied the anti and health-aging of tomato using *Drosophila melanogaster* as an aging animal model. Tomato and lycopene decreased both average lifespan and average healthspan at the highest assayed concentrations. DAI et al. (2008) observed that lycopene increased the average lifespan in male and female flies at 2.5 and 7.5 μg/g. We disagree with these results. This discrepancy could be due to the different concentrations used. Furthermore, dietary supplementation does not always result in an increased life span (Soh et al., 2007; Tasset-Cuevas et al., 2013).

When a substance is considered as an anti-genotoxic agent, generally exhibit chemotherapeutic effects that could be used in the strategy of cancer control. Anti-proliferative, proapoptotic and DNA strand break capacities are suitable when the healthy effect of antioxidant is assessed. Our anti-proliferative activity result was promising for tomato in HL60 cancer cell model and was in agreement with those results obtained by other authors: i) Friedman et al. (2009) determined that different tomato extracts had anti-proliferative effect on stomach, liver, breast and colon cancer cell lines using MTT assay and ii) Ferreres et al. (2010) showed that tomato seed aqueous extract displayed a concentration-dependent cell proliferation inhibition against rat basophile leukaemia (RBL-2H3) cell line using SBR assay. Although the IC_{50} obtained by Ferreres et al. (2010) was higher (5.9 mg/mL) than the IC_{50} obtained by our (0.75 mg/mL). This discrepancy could be due to the different sample, genetic background and assay used.

In contrast, lycopene had not cytotoxic effect on HL60 cell line. This result is in concordance with those obtained by other authors (Hantz et al., 2005; Kotake-Nara et al., 2001). Nevertheless, Amir et al. (1999) demonstrated that lycopene had a concentration-dependent cytotoxic effect in HL-60 cell growth as measured by [3H]thymidine incorporation.

Salman et al. (2007) and Teodoro et al. (2012) reported that the anti-proliferative effect of lycopene on tumour cell and its effect on the apoptotic rate depends on its dosage and on the type of malignant cells.

Furthermore, due to the tomato is considered a complex mixture; it is not easy to establish which compounds are responsible for the anti-proliferative effect on HL60 cell line.

To our knowledge, it is the first time that it is studied the pro-apoptotic effect by fresh tomato using DNA fragmentation method. Neither tomato nor lycopene showed the typical inter-nucleosomic fragment pattern of pro-apoptotic mechanism. Nevertheless, Hwang and Bowen (2004) observed that tomato paste hexane extract at 5 µM induced apoptosis in prostate cancer cell line (LNCaP) at the late stages of the 24 and 48 h of treatment. This discrepancy could be due to the different cancer cell line used and the different time of treatment. Respect to the induction of DNA-fragmentation by lycopene in tumour cells, the results yielded in other researches are different depending of the cell line, time of treatment and dosages: Salman et al. (2007) reported that lycopene caused an enhancement of the apoptotic rate of human colon carcinoma and Raji cells at different concentrations (1, 2 and 4 µM for colon carcinoma and

only 4 µM for Raji). On the other hand, these authors showed that lycopene did not caused apoptosis in human erythroleukaemia and B chronic lymphocytic leukaemia cells. Hantz et al. (2005) also demonstrated that lycopene (0.3, 1 and 3 µM) induced DNA fragmentation in LNCaP human prostate cancer cells at the 72 h of treatment but did not happen at the 20 h of treatment.

Apoptotic mechanism can be occurring in the absence of DNA internucleosomic fragment (Cohen et al., 1992). For this reason, we performed alkaline SCGE or comet assay in order to detect DNA strand breaks, which could help to determine if the cell death occurs against apoptotic or necrotic pathway. This hallmark has been used for the screening of substances with single DNA-strand break activity for to be used in the leukaemia treatment (Yedjou and Tchounwou, 2007). The TM parameter is widely used to differentiate apoptosis-from necrosis-induced DNA damage: Different values have been ascribe to TM parameter: TM >30 (hedgehog pattern) indicates apoptotic processes, necrotic processes have a short comet-tail moment where the highest amount of damaged DNA remains in the comet head (Fairbairn and O'Neill, 1995). Our results showed that the tomato and lycopene DNA-induced damage was probably mediated by a necrotic mechanism in HL60 cell line (TM lower than 3). These results are in concordance with our anti-proliferative and pro-apoptotic assays, demonstrating that tomato and lycopene cause an anti-proliferative effect in HL60 by a necrotic pathway because they had the same DNA damage pattern (class 1, 1<TM<5 according to Fabiani et al. (2012)). To our knowledge, no report exits on DNA-induced damage against

lycopene-induced DNA strand breaks in tumour cells. Respecto to the lycopene-induced DNA damage, we disagree with the results obtained by Park et al. (2005), who reported that supplementation with lycopene during 24 h of treatment induced DNA strand breaks (oxidative DNA damage) in the human hepatoma cell line (Hep3B). This discrepancy could be due to the different time of treatment and cell line used.

The different assay carried out to determine the biological activity of food or medicinal plants, the distinctive compounds present in the complex matrix (the food) mimic the whole food activity. In the case of lycopene, this rule is applied.

Based on the findings of the present study, we conclude that i) Both tomato and lycopene are safe (no genotoxic) and antimutagenic compared to hydrogen peroxide. Although lycopene is always lesser extend; ii) Respect to the lifespan, tomato and lycopene are harmless at the lowest concentration. In general, both lifespan and healthspan reductions of Drosophila are highest in the case of lycopene; iii) Tomato is cytotoxic in a dose-dependent manner, lycopene although it is not; iv) Tomato and lycopene do not induce DNA-fragmentation but they induce significant clastogenic activity at low level.

Taking into account the protection of the DNA damage and the possible chemo-preventive activity, tomato is the best candidate to be considered as nutraceutical substance. We have a case where the additive or synergistic action of other components within of the tomato matrix could

be the cause of the health effects observed in this vegetable, which are not fully explained by lycopene.

REFERENCES

Abraham, S.K., 1994. Antigenotoxicity of coffee in the Drosophila assay for somatic mutation and recombination. Mutagenesis 9, 383-386.

Agarwal, S., Rao, A.V., 2000. Tomato lycopene and its role in human health and chronic diseases. Can. Med. Assoc. J. 163, 739-744.

Agrawal, R., Jain, R., Raja, W., Ovais, M., 2009. Anticarcinogenic effects of Solanum lycopersicum fruit extract on Swiss albino and C57 Bl mice. Asian Pac. J. Cancer P. 10, 379-381.

Amir, H., Karas, M., Giat, J., Danilenko, M., Levy, R., Yermiahu, T., Levy, J., Sharoni, Y., 1999. Lycopene and 1, 25-dihydroxyvitamin d3 cooperate in the inhibition of cell cycle progression and induction of differentiation in hl-60 leukemic cells.

Anh, N.T.T., Nishitani, M., Harada, S., Yamaguchi, M., Kamei, K., 2011. A Drosophila model for the screening of bioavailable NADPH oxidase inhibitors and antioxidants. Mol. Cell Biochem. 352, 91-98.

Asita, A.O., Dingann, M.E., Magama, S., 2008. Lack of modulatory effect of asparagus, tomato, and grape juice on cyclophosphamide-induced genotoxicity in mice. Afr. J. Biot. 7.

Benzie, I.F., 2003. Evolution of dietary antioxidants. Comp. Biochem. Phys. A 136, 113-126.

Bhuvaneswari, V., Velmurugan, B., Abraham, S., Nagini, S., 2004. Tomato and garlic by gavage modulate 7, 12-dimethylbenz [a] anthracene-induced genotoxicity and oxidative stress in mice. Braz. J. Med. Biol. Res. 37, 1029-1034.

Canene-Adams, K., Campbell, J.K., Zaripheh, S., Jeffery, E.H., Erdman, J.W., 2005. The tomato as a functional food. J. Nutr. 135, 1226-1230.

Cohen, G., Sun, X., Snowden, R., Dinsdale, D., Skilleter, D., 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem. J. 286, 331-334.

DAI, Q.-p., WANG, L.-f., GUO, W.-w., WANG, F.-h., ZHANG, X.-w., XU, S.-h., 2008. EFFECT OF LYCOPENE ON LIFESPAN OF DROSOPHILA AND ITS UNDERLYING MOLECULAR MECHANISM [J]. Acta Nutrimenta Sinica 4, 020.

Di Mascio, P., Kaiser, S., Sies, H., 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch. Biochem. Biophys 274, 532-538.

Dutra, E.S., Dias, C.D., Araújo, B.C.d., Castro, A.J., Nepomuceno, J.C., 2009. Effect of organic tomato (Lycopersicon esculentum) extract on the genotoxicity of doxorubicin in the Drosophila wing spot test. Genet. Mol. Biol. 32, 133-137.

Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., Morozzi, G., 2012. Genotoxicity of alkene epoxides in human peripheral blood mononuclear cells and HL60 leukaemia cells evaluated with the comet assay. Mutat. Res. 747, 1-6.

Fairbairn, D.W., O'Neill, K.L., 1995. Necrotic DNA degradation mimics apoptotic nucleosomal fragmentation comet tail length. In Vitro Cell.Dev.-An. 31, 171-173.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Ferreres, F., Taveira, M., Pereira, D.M., Valentão, P.c., B. Andrade, P., 2010. Tomato (Lycopersicon esculentum) seeds: new flavonols and cytotoxic effect. J. Agr. Food Chem. 58, 2854-2861.

Frei, H., Würgler, F.E., 1995. Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in Drosophila. Mutat. Res. 334, 247-258.

Friedman, M., Levin, C.E., Lee, S.-U., Kim, H.-J., Lee, I.-S., Byun, J.-O., Kozukue, N., 2009. Tomatine-containing green tomato extracts inhibit growth of human breast, colon, liver, and stomach cancer cells. J. Agr. Food Chem. 57, 5727-5733.

George, B., Kaur, C., Khurdiya, D., Kapoor, H., 2004. Antioxidants in tomato (Lycopersium esculentum) as a function of genotype. Food Chem. 84, 45-51.

Graf, U., Abraham, S.K., Guzmán-Rincón, J., Würgler, F.E., 1998. Antigenotoxicity studies in Drosophila melanogaster. Mutat. Res.- Fund. Mol. M. 402, 203-209.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Gyori, B.M., Venkatachalam, G., Thiagarajan, P.S., Hsu, D., Clement, M.V., 2014. OpenComet: An automated tool for comet assay image analysis. Redox Biol. 2, 457-465.

Hantz, H.L., Young, L.F., Martin, K.R., 2005. Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. Exp. Biol. Med. 230, 171-179.

Hu, W., Dai, D., Li, W., 2013. Extension of Life Span and Improvement of Vitality in Drosophila melanogaster by Supplementation with Lycopene Mycelium Powder from Blakeslea trispora. Food Sci. Technol. Res. 19, 513-518.

Hwang, E., Bowen, P., 2004. Effects of tomato paste extracts on cell proliferation, cell-cycle arrest and apoptosis in LNCaP human prostate cancer cells. BioFactors (Oxford, England) 23, 75-84.

Jones, M.A., Grotewiel, M., 2011. Drosophila as a model for age-related impairment in locomotor and other behaviors. Exp. Gerontol. 46, 320-325.

Klonoff, D.C., 2009. The beneficial effects of a Paleolithic diet on type 2 diabetes and other risk factors for cardiovascular disease. J. Diabetes Sci. Technol. 3, 1229.

Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., Nagao, A., 2001. Carotenoids affect proliferation of human prostate cancer cells. J. Nutr. 131, 3303-3306.

Lindeberg, S., Cordain, L., Eaton, S.B., 2003. Biological and clinical potential of a palaeolithic diet. J. Nutr. Environ. Med. 13, 149-160.

Lugasi, A., Bíró, L., Hóvárie, J., Sági, K.V., Brandt, S., Barna, É., 2003. Lycopene content of foods and lycopene intake in two groups of the Hungarian population. Nutr. Res. 23, 1035-1044.

McClain, R.M., Bausch, J., 2003. Summary of safety studies conducted with synthetic lycopene. Regul. Toxicol. Pharm. 37, 274-285.

Minois, N., 2006. How should we assess the impact of genetic changes on ageing in model species? Ageing Res. Rev. 5, 52-59.

Olive, P.L., Banáth, J.P., 2006. The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1, 23.

Palozza, P., Bellovino, D., Simone, R., Boninsegna, A., Cellini, F., Monastra, G., Gaetani, S., 2009. Effect of β-carotene-rich tomato lycopene β-cyclase (tlcy-b) on cell growth inhibition in HT-29 colon adenocarcinoma cells. Brit. J. Nutr. 102, 207-214.

Park, Y.O., Hwang, E.S., Moon, T.W., 2005. The effect of lycopene on cell growth and oxidative DNA damage of Hep3B human hepatoma cells. Biofactors 23, 129-139.

Peralta, I.E., Spooner, D.M., 2006. History, origin and early cultivation of tomato (Solanaceae). Genetic improvement of Solanaceous crops 2, 1-27.

Rauscher, R., Edenharder, R., Platt, K., 1998. In vitro antimutagenic and in vivo anticlastogenic effects of carotenoids and solvent extracts from fruits and vegetables rich in carotenoids. Mutat. Res. 413, 129-142.

Reddy, L., Odhav, B., Bhoola, K., 2006. Aflatoxin B1-induced toxicity in HepG2 cells inhibited by carotenoids: morphology, apoptosis and DNA damage. Biol. Chem. 387, 87-93.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., Bier, E., 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res. 11, 1114-1125.

Ren, N., Charlton, J., Adler, P.N., 2007. The flare gene, which encodes the AIP1 protein of Drosophila, functions to regulate F-actin disassembly in pupal epidermal cells. Genetics 176, 2223-2234.

Rodríguez-Muñoz, E., Herrera-Ruiz, G., Pedraza-Aboytes, G., Loarca-Piña, G., 2009. Antioxidant capacity and antimutagenic activity of natural oleoresin from greenhouse grown tomatoes (Lycopersicon esculentum). Plant Foods Hum. Nutr. 64, 46-51.

Rojas-Molina, M., Campos-Sánchez, J., Analla, M., Muñoz-Serrano, A., Alonso-Moraga, Á., 2005. Genotoxicity of vegetable cooking oils in the Drosophila wing spot test. Environ. Mol. Mutagen. 45, 90-95.

Romero-Jimenez, M., Campos-Sanchez, J., Analla, M., Muñoz-Serrano, A., Alonso-Moraga, Á., 2005. Genotoxicity and anti-genotoxicity of some traditional medicinal herbs. Mutat. Res. 585, 147-155.

Salman, H., Bergman, M., Djaldetti, M., Bessler, H., 2007. Lycopene affects proliferation and apoptosis of four malignant cell lines. Biomed. Pharmacother. 61, 366-369.

Scolastici, C., de Lima, R.A., Barbisan, L., Ferreira, A., Ribeiro, D., Salvadori, D.M.F., 2008. Antigenotoxicity and antimutagenicity of lycopene in HepG2 cell line evaluated by the comet assay and micronucleus test. Toxicol. in Vitro 22, 510-514.

Serpeloni, J.M., dos Reis, M.B., Rodrigues, J., dos Santos, L.C., Vilegas, W., Varanda, E.A., Dokkedal, A.L., Cólus, I.M.S., 2008. In vivo assessment of DNA damage and protective effects of extracts from Miconia species using the comet assay and micronucleus test. Mutagenesis 23, 501-507.

Silaste, M.-L., Alfthan, G., Aro, A., Antero Kesäniemi, Y., Hörkkö, S., 2007. Tomato juice decreases LDL cholesterol levels and increases LDL resistance to oxidation. Brit. J. Nutr. 98, 1251-1258.

Soh, J.W., Hotic, S., Arking, R., 2007. Dietary restriction in Drosophila is dependent on mitochondrial efficiency and constrained by pre-existing extended longevity. Mech. Aging Dev. 128, 581-593.

Tasset-Cuevas, I., Fernández-Bedmar, Z., Lozano-Baena, M.D., Campos-Sánchez, J., de Haro-Bailón, A., Muñoz-Serrano, A., Alonso-Moraga, Á., 2013. Protective effect of borage seed oil and gamma linolenic acid on DNA: in vivo and in vitro studies. Plos One 8, e56986.

Teodoro, A.J., Oliveira, F.L., Martins, N.B., Maia, G.d.A., Martucci, R.B., Borojevic, R., 2012. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. Cancer Cell. Int. 12, 36.

Toor, R.K., Savage, G.P., 2005. Antioxidant activity in different fractions of tomatoes. Food Res. Int. 38, 487-494.

Velmurugan, B., Bhuvaneswari, V., Abraham, S.K., Nagini, S., 2004a. Protective effect of tomato against N-methyl-N'-nitro-N-nitrosoguanidine–induced in vivo clastogenicity and oxidative stress. Nutrition 20, 812-816.

Velmurugan, B., Santhiya, S.T., Nagini, S., 2004b. Protective effect of S-allylcysteine and lycopene in combination against N-methyl-N'-nitro-N-nitrosoguanidine-induced genotoxicity. Pol. J. Pharmacol. 56, 241-246.

Verhoeyen, M., Bovy, A., Collins, G., Muir, S., Robinson, S., De Vos, C., Colliver, S., 2002. Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. J. Exp. Bot. 53, 2099-2106.

Who, J., Consultation, F.E., 2003. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser 916.

Willcox, J.K., Catignani, G.L., Lazarus, S., 2003. Tomatoes and cardiovascular health.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., Adler, P.N., 2008. The multiple-wing-hairs gene encodes a novel GBD–FH3 domain-containing protein that functions both prior to and after wing hair initiation. Genetics 180, 219-228.

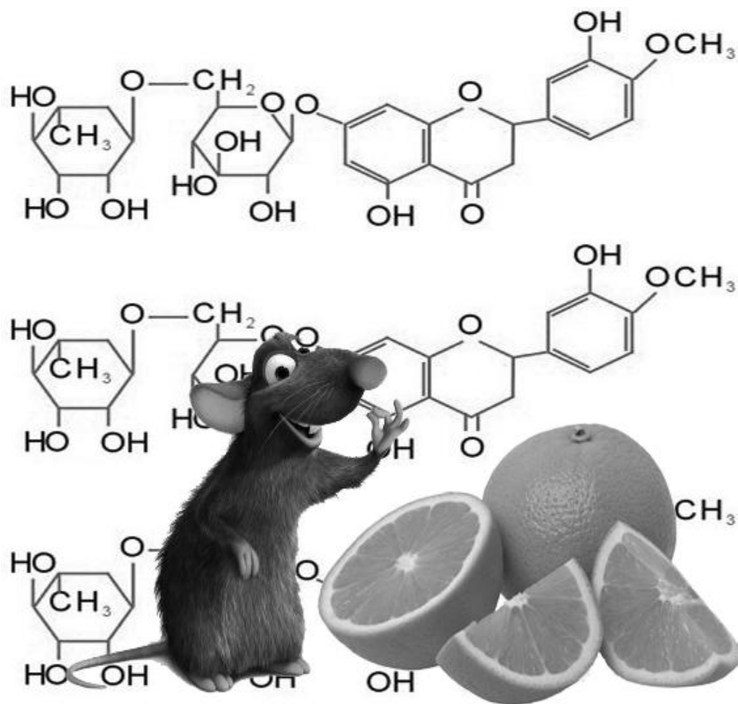
Yedjou, C.G., Tchounwou, P.B., 2007. In-vitro cytotoxic and genotoxic effects of arsenic trioxide on human leukemia (HL-60) cells using the MTT and alkaline single cell gel electrophoresis (Comet) assays. Mol. Cell Biochem. 301, 123-130.

Zhang, Z., Han, S., Wang, H., Wang, T., 2014. Lutein extends the lifespan of Drosophila melanogaster. Arch. Gerontol. Geriat. 58, 153-159.

CAPÍTULO V: Demethylation and Anticarcinogenic Potency of Hesperidin *In vitro* and *In vivo* Assays.

Chapter V

Artículo en preparación



ABSTRACT

Hepatocellular carcinomas (HCC) is one of the most common neoplasia representing the fifth most common malignancy worldwide and the third most common cause of death from cancer. Diets high in fruits and vegetables are widely recommended for their health-promoting properties, among them, the protection against diabetes, cancer and cardiovascular diseases. Studies have been carried out using diet components as possible modulator agents of DNA methylation in cancer cells. Epigenetic therapy against harmful effects of diet components could be a potential tool in chemotherapy. Hesperidin is the most important phenol in the orange fruit and its biological healthy activities are well known. The purpose of the present study was to evaluate the methylation patterns induced by hesperidin in Hl60 *in vitro* model cell line in order be used as a chemopreventive molecule in epigenetic cancer therapies. An *in vivo* pilot experience using a rat diethyl nitrosamine hepatocarcinogenesis-induced model is carried out to validate the therapeutic efficacy of this orange flavonol. Results showed that: (i) Hesperidin is cytotoxic in a dose-dependent manner and the IC_{50} was 12.5 mM; (ii) Hesperidin has a dose dependent hypomethylating effect in the LINE-1 sequence (up to 47% hypomethylation at 12.5 mM) and in the ALU repetitive sequences (up to 32 % at 6 mM) in HL60 tumour cells. (iii) Hesperidin do not affect to the rat body and liver weight and it reduces the DEN-induced nodules at 1000 ppm. In conclusion, hesperidin could be purpose as candidate molecule in chemoprevention against epigenetic therapy.

KEY WORDS: hesperidin, hepatocarcinogenesis, rat, HL-60 promyelocytic cells, methylation, diethylnitrosamine.

INTRODUCTION

Diets high in fruits and vegetables are widely recommended for their health-promoting properties. In particular, oranges have health benefits including protection against diabetes, cancer and cardiovascular diseases (da Silva, 2005; González-Molina et al., 2010; So et al., 1996). The great contributors to these beneficial effects are the orange polyphenols (Bishayee et al., 2012; Vinson et al., 2002). Oranges contain a large array of polyphenolic constituents: hydroxycinnamic acids as caffeic, p-coumaric, and ferulic sinapic and flavonoids, among which narirutin, hesperidin didymin are predominant (Klimczak et al., 2007) as other components that have demonstrated to be potential chemopreventive agents in epidemiologic, *in vivo* and *in vitro* studies (Fernández-Bedmar et al., 2011). Among them, hesperidin (a flavanone glycoside, $C_{28}H_{34}O_{15}$) is the most distinctive component of *Citrus* fruit and juice (Garg et al., 2001; Gattuso et al., 2007) that is hydrolysed by gut microflora into aglycone form (hesperetin) (Vallejo et al., 2010). Although research on the health effects of hesperidin is fairly limited, this flavonoid has demonstrated to be responsible for many biological activities and exhibits a high number of biological and pharmacological properties such as anti-inflammatory, anti-carcinogenic, antioxidative, and lipid-lowering activities (Chiba et al., 2003; Middleton Jr and Kandaswami, 1994; Miyake et al., 1998). Moreover, recent studies carried out in our laboratory demonstrated that hesperidin has an antigenotoxic effect against H_2O_2-DNA induced damage and a chemopreventive activity on HL60 cells (Fernández-Bedmar et al., 2011).

In relation to anticarcinogenic effects of hesperidin, some studies have shown that this polyphenol can inhibit carcinogenesis in distinct cancer types (tongue, esophagous, colon…) (Kelloff et al., 1994; Tanaka et al., 1997b; Tanaka and Sugie, 2007). However, no studies have been reported about anticarcinogenic effect of hesperidin in liver. Hepatocellular carcinoma (HCC) is one of the most common neoplasias representing the fifth most common malignancy worldwide and the third most common cause of death from cancer (Forner and Bruix, 2012). Currently, surgical resection and liver transplantation are the only treatment options for HCC (Forner and Bruix, 2012; Pang et al., 2006; Singh et al., 2014). For this reason, it is important to focus on chemopreventive strategies to prevent the development of this malignancy although this matter is still fairly limited.

At the molecular level, cancer can be considered a result of abnormal genetic and epigenetic events. The epigenetic processes are responsible for activate or inactivate selectively the gene function. DNA methylation is an important epigenetic mechanism of cancer development and progression. Components present in the diet can affect DNA methylation and thus cancer risk and tumour behaviour (Davis and Uthus, 2004). In particular, common dietary phenolic compounds among them, the hesperetin flavanol, inhibit the DNA methyltransferase (DNMT) activity *in vitro* (Li and Tollefsbol, 2010). DNA methylation in mammalian cells, occurs at 5´ cytosine residues within CpG dinucleotides through addition of CH_3 group to form 5-methylcytosine (Issa and Kantarjian, 2009). The human genome have not uniformly distributed the CpG dinucleotides, but they are often

enriched in the promoter regions of genes, as well as regions of large repetitive sequences (e.g. centromeric repeats, LINE and ALU retrotransposon elements) (Bird, 2002). Short CpG-rich regions are known as ''CpG islands'', and these are present in more than 50% of human gene promoters. The inhibitory activity of dietary polyphenols has been associated with the demethylation of these CpG islands in the promoters (Fang et al., 2007). Since epigenetic changes are reversible they are promising targets for chemotherapy using agents, such as dietary polyphenols, that inhibit DNA methylation (Link et al., 2010).

Thus, the purpose of the present study was, first, to evaluate the methylation patterns induced by hesperidin in Hl60 *in vitro* model cell line and second, validate the therapeutic efficacy of this orange flavonol in an *in vivo* pilot experience using a rat diethyl nitrosamine hepatocarcinogenesis-induced model.

MATERIAL AND METHODS

Chemicals

Hesperidin (H5254) as a single compound contained in the orange fruit and diethylnitrosamine (DEN) (N0756) as the hepatocarcinogen inductor were purchased from Sigma (Córdoba-Spain).

In vitro demethylating study

Cell culture conditions

The HL-60 human leukaemia cancer cell line was provided by Dr. Villalba (Department of Cell Biology, University of Córdoba, Spain). HL60 cells were

grown in RPMI-1640 medium (Sigma, R5886) supplemented with L-glutamine 200 mM, (Sigma, G7513), antibiotic-antimycotic solution (Sigma, A5955) and 10% heat-inactivated foetal bovine serum (Linus, S01805). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The cultures were plated at 2.5 x 10^4cells/ml density in 10ml culture bottles and passed every 2 days.

Cytotoxicity control assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4cells/ml) and treated for 72h with a wide range of hesperidin concentration (from 0.78 to 25 mM) in order to assess the cytotoxic doses ranging the inhibitory concentration 50 (IC_{50}). This assessment with a wide range of concentrations in the *in vitro* cytotoxicity assays was sat with the aim to choose the appropriate *in vivo* lethality doses to be used in the subsequent methylation assay.

Cell viability was determined by the trypan blue dye (Sigma, T8154) exclusion test. After incubation period, cells were stained with Trypan Blue dye (1:1 v/v) and counted under inverted microscope using a Neubauer chamber at 100X magnification. Curves were plotted as a survival percentage of each treatment compared with the control growing at 72h. At least three independent repetitions of the assays were carried out to calculate means for statistical analysis.

HL60 global DNA methylation evaluation

DNA isolation and bisulfite treatment

In order to study the global methylation status of the HL60 leukemic cell genome, HL60 cells (1.5 x 10^6 cells/mL) were treated for 72 h with supplemented medium containing the different tested concentrations of hesperidin (0.78, 3, 6,12.5 and 25 mM). After the treatment period, the genomic DNA of HL60 cells was isolated using a commercial DNA-extraction kit following the manufacturer instructions (Blood Genomic DNA Extraction Mini Spin Kit, Canvax Biotech, Córdoba, Spain). Genomic DNA (1µg) was denatured with NaOH and then treated with bisulfite using a commercial kit (CpGENOME^{TM} DNA MODIFICATION KIT, S7820, Chemicon international). This bisulfite treatment converts unmethylated cytosine residues to uracil, whereas 5-methylcytosine residues remain unchanged.

Quantitative real-time methylation-specific PCR (qrt-MSP) of repetitive sequences

Analysis of changes in DNA methylation after different hesperidin treatment was carried out in a rapid fluorescent thermal cycler with three-colour fluorescence monitoring capability (LightCycler, Roche) according to manufacturer´s protocol (LightCycler^{R}FastStart DNA Master^{Plus} SybrGreen I, Cat. No. 03515885001, Roche) and following the method described by Fernández-Bedmar et al., (2012). Briefly, PCR was performed whit 1 µL of bisulfite treated DNA using specific primers for LINE1, Satellite-α (SATα), Satellite-2 (SAT2) and ALU-M2 (Table 1).

Table 1. Information of the primers employed in this study

Primer	Forward Sequence (F) 5´-3´	Reverse Sequence (R) 5´- 3´	GC content (%)	
			F	R
ALU-C4	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA	ATTAACTAAACTAATCTTAAACTCCTAACCTCA	25	27.3
LINE-1-M1	GGACGTATTTGGAAAATCGGG	AATCTCGCGATACGCCGTT	47.6	52.6
SAT-α-M1	TGATGGAGTATTTTTAAAATATACGTTTTGTAGT	AATTCTAAAAATATTCCTCTTCAATTACGTAAA	23.5	21.2
SAT-2-M1	TCGAATGGAATTAATATTTAACGGAAAA	CCATTCGAATCCATTCGATAATTCT	25	36
ALU-M2	GCGCGGTGGTTTACGTTT	AACCGAACTAATCTCGAACTCCTAAC	55.5	42.3

The final reaction mix with a total volume of 10µl consisted of: 1 µL of bisulfite converted genomic DNA, 5 µl of milliQ water, 1 µl of each primer at 0.5µM (Isogen Life Science BV) and 2µl of Master Mix reaction (Roche, Cat.No. 03515885001, LightCycler^{R} FastStart DNA Master^{PLUS} SybrGreen I Master Mix, 5x). Amplification of the repetitive methylated sequences was used as target sequences. The methylated reaction specific for the LINE-1 and ALU-M2 sequences were based on a LINE-1 (GenBank accession number X52235 and ALU-M2 consensus sequences. The Sat-α and Sat-2 reactions were designed toward sequences specifically on chromosome 1 (GenBank accession numbers M38468 and X72623, respectively)

The following program conditions were applied for qrt-MSP running: denaturation program, consisting in 1 cycle at 95C for 10 min; amplification program, consisting in 45 cycles at 95°C for 10 s, 65°C for 10 s and 72°C for

10 s; melting program, 1 cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling program, 1 cycle at 40°C for 60 s. The temperature transition rate was 20°C /s, except in the melting program, which was 0.4°C /s between 40°C and 90°C. Amplification of an Alu-control sequence (Alu-C4) which is not modified by bisulfite treatment was performed as reference sequence (Weisenberger et al., 2005). It was amplified in the same run and following the same procedure described above for the different unmethylated repetitive sequences. A procedure based on the relative quantification of target sequence (Alu, LINE1, Sat-α and Sat-2 unmethylated sequences) versus their controls/calibrators in relation to the reference sequence (Alu-C4) was used to assess the degree of repetitive elements hypomethylation. Calculations were automatically performed by LightCycler software (LyghtCycler software, version 4.05, Roche). The normalised ratio was obtained from the next equation and expressed as percentage of the control/calibrator:

Normalised ratio $(N_{repetitive}) = (E_{target})^{\Delta Cp\ target\ (control–sample)} / (E_{ref})^{\Delta Cp\ ref\ (control–sample)}$

Where E indicates efficiencies of each sequence calculated from the slopes of crossover points (C_P) versus DNA concentration plot according to the formula $E = 10^{(-1/slope)}$; ΔCp indicates the difference between control/calibrator Cp and sample Cp, either for the target or for the reference sequences. Water blank and negative controls were included. Results were confirmed by repeating the bisulfite treatment and MSP assays for all samples.

***In vivo* study**

Animals and diet

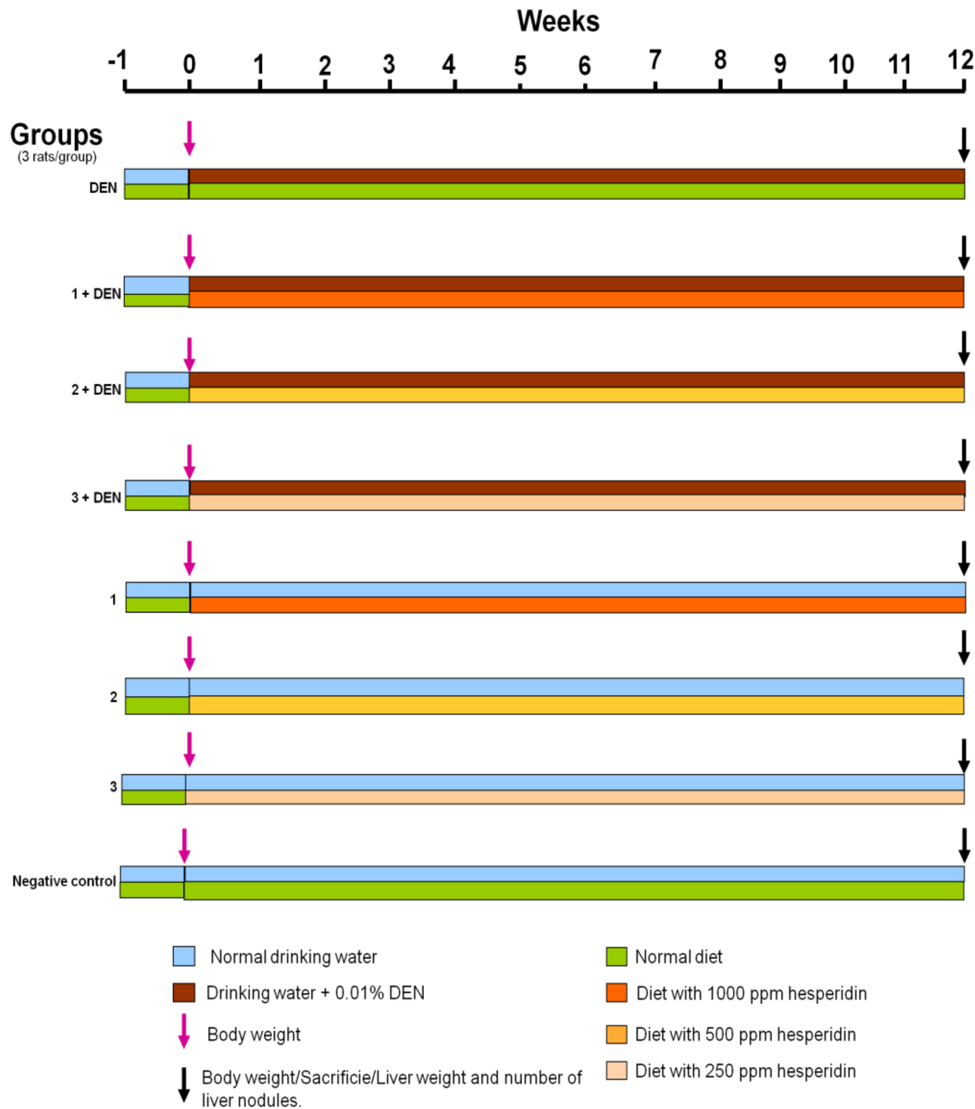
Twenty-four pathogen-free male Sprague-Dawley rats (324 gr ± 25 gr), seven weeks old, were purchased from Harlan Interfaunan (Iberica) S.L and housed in a conventional metabolic cage. The rats were acclimatized for one week previous to the experiment, with controlled temperature (23º ± 2ºC) and humidity (55% ± 5%). The animals were fed with a standard diet (D03- SAFE, Augy, France) and provided with drinking water ad libitum. Animal care and experimental procedures were approved by the University of Cordoba Bioethics Committee, and followed the regulations of the European Union normative for care and use of laboratory animals.

Experimental design

After one week acclimatization, twenty-four rats were randomly divided into eight groups as follows: 1) Negative control group (rats with standard diet and water ad libitum). 2) Positive control group (rats with standard diet and DEN diluted at 0.01% in drinking water). 3) Group 1 (rats with standard diet supplemented with 1000 ppm of hesperidin. 4) Group 2 (rats with standard diet supplemented with 500 ppm of hesperidin. 5) Group 3 (rats with standard diet supplemented with 250 ppm of hesperidin. 6) Group 1 + DEN (rats with standard diet supplemented with 1000 ppm of hesperidin and 0.01% DEN in drinking water. 7) Group 2 + DEN (rats with standard diet supplemented with 500 ppm of hesperidin and 0.01% DEN in drinking water). 8) Group 3 + DEN (rats with standard diet supplemented

with 250 ppm of hesperidin and 0.01% DEN in drinking water). A schematic representation of the study design is provided in the **Figure 1**.

Figure 1. Hepatocarcinogenic experimental design in rats treated with DEN and hesperidin.



Histopathological study: macroscopic and microscopic study.

At the end of the study (12 weeks), the rats were weighed and sacrificed by carbon dioxide chamber and posterior decapitation. The livers from all animals were perfused through the portal vein with saline solution and subsequently excised, weighed and photographed. Each liver was examined macroscopically on the surface and in 3 mm cross-sections for gross visible nodules and percentage of affectation. Measurements of nodules were done in two perpendicular planes to obtain an average diameter of each nodule. The nodules were categorized into three groups (≥3, <3->1 and ≤ 1 mm) according to Bishayee and Dhir (2009).

For microscopy study, representative sections from right, left and caudate lobes of each liver were taken, as well as the largest lesions found, and fixed in buffered formalin (10%), embedded in paraffin wax and stored at 4ºC. Sections were stained with hematoxylin and eosin. Hepatic lesions were classified by light microscopy by two different exposure-blinded pathologists according the guidelines proposed by Thoolen et al. (2010). Additionally, mitotic figures were counted in 10 non-overlapping high power fields per sample.

Statistical analysis

The results are expressed as mean ± SD. For statistical data evaluation, the software GraphPad PRISM 5 version 5.01 (GraphPad Software Inc, San Diego, CA, USA). was used. Differences between the means of groups were assessed by paired t-test when data were normally distributed and

otherwise by the Wilcoxon test. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

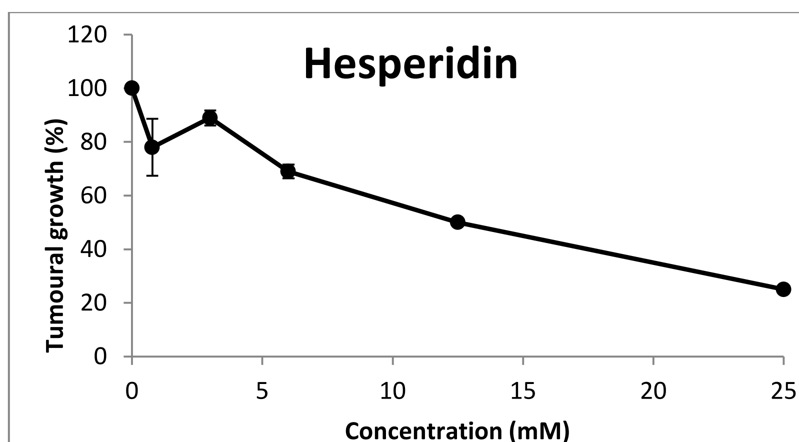
In vitro study

Optimal cytotoxic concentrations of hesperidin

In order to choose the correct cytotoxic concentrations to carry out the global methylation evaluation, a control assay was performed based on the hesperidin cytotoxic effect on the HL60 cell line.

Figure 2 shows the cytotoxic effect of hesperidin at the different assayed concentrations. Hesperidin exerted a positive dose-dependent cytotoxic effect as expected and the inhibition concentration 50 was established at 12.5 mM. As a consequence, the concentrations 0.78, 3, 6, 12.5 and 25 mM were chosen for the methylation checking.

Figure 2. Cytotoxic effect of hesperidin on HL60 cells after 72 hours treatment.

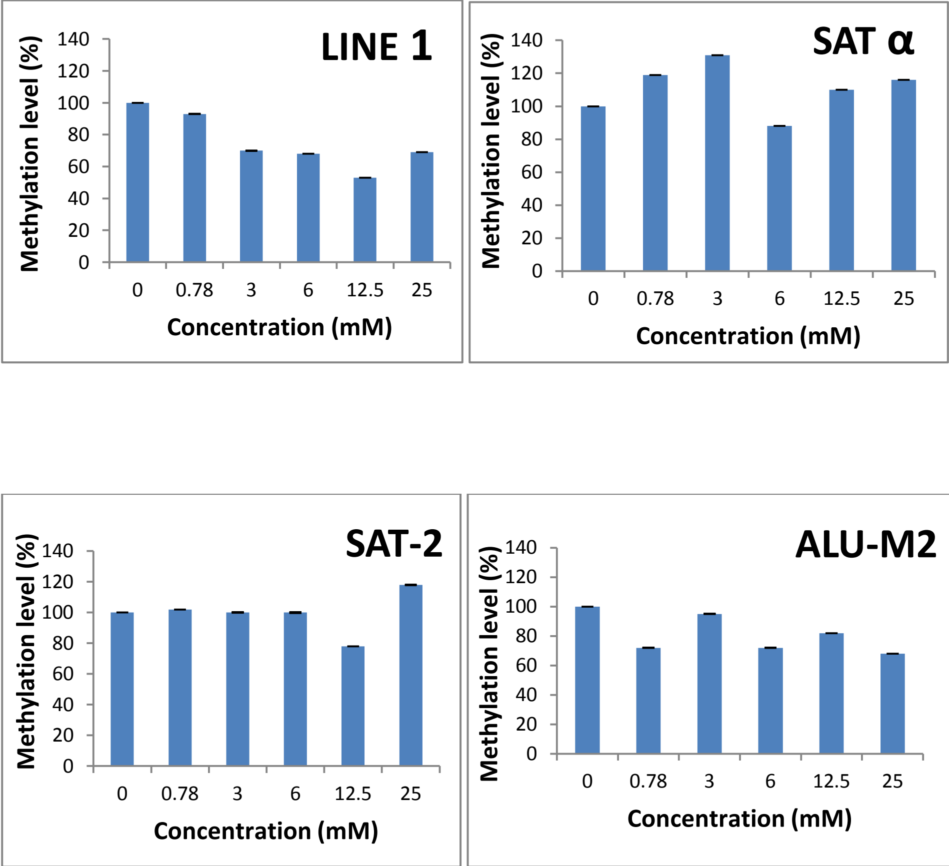


Global DNA methylation evaluation

Figure 3 shows the methylation status of the four repetitive sequences studied in the HL60 genome at the five different tested concentrations of hesperidin (0.78, 3, 6, 12.5 and 25 mM).

Hesperidin induced an increase of the methylation status in the repetitive sequence SAT-α compared with their relative control at all tested concentrations (0.78, 3, 12.5 and 25 mM) except for 6mM that it induced an decrease of the methylation (hypomethylation) by 12 %; No dose-effect is observed but a methylation average of 112.8% was observed. SAT-2 sequence showed maintenance of the methylation status at the lowest concentrations (0.78, 3 and 6 mM), although hesperidin at 12.5 mM induced a hypomethylation by 22% for this repetitive element, yielding a methylation average of 99.6 %. Hesperidin induced hypomethylation at all tested concentrations in LINE1. A dose-depend hypomethylation is observed for this repetitive sequence (from 0.78 to 12.5 Mm) by 7%, 30%, 32% and 47 % respectively. The highest concentration of hesperidin (25mM) also demethylated the LINE1 promoter at the same percentage that 6 mM concentration (32%). The methylation average for this repetitive sequence was 70.6%. All tested concentrations of hesperidin exhibited a demethylation activity for ALU-M2 although different levels of hypomethylation were observed in this repetitive sequence being at 25 mM the highest value obtained by 32%. ALU-M2 yielded a methylated average of 77.8 %.

Figure 3. Repetitive DNA methylation in HL60 genome after 72 hours of hesperidin treatment.



***In vivo* study**

Effect of hesperidin on body and relative liver weights.

Hesperidin did not interfere with the animal´s growth since there was no statistical difference in the average body weights of groups treated with

hesperidin alone (Groups 1, 2 and 3) as well as in the negative control group (**Table 2**). However, DEN treatment (positive control group) significantly decreased the body weights of rats as compared with negative control group at the end of the experiment (**Table 2, Figure 4**). Regarding groups where DEN was supplemented with hesperidin, no significant changes were observed on body weight. (**Table 2, Figure 4**).

The average liver weight of DEN group was significantly increased as compared to that of negative control group. Nevertheless, neither hesperidin alone nor combined with DEN at different doses significantly altered the liver weights. (**Table 2, Figure 5**).

Table 2. Initial and final body weight, liver weight and total number of liver nodules found in the different groups of rats. Values are presented as means±SD. *P≤0.05

Groups	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Total no. of nodules
Control	274.3±11.3	453.0±13.2	14.0±3.3	0±0
1	270.7±13.6	447.3±8.5	14.3±01.6	0±0
2	281.0±16.8	459.7±17.0	15.2±1.2	0±0
3	268.7±14.4	448.0±12.1	14.5±1.5	0±0
DEN	275.3±14.8	379.3±15.5*	19.5±3.9*	112.7±23.3
1 + DEN	266.0±21.1	379.3±3.0	17.1±2.8	25.0±17.6*
2 + DEN	286.3±20	328.7±45.7	16.6±4	79.3±3.4
3 + DEN	274.3±2.5	380.7±18.3	20.6±6.2	51.6±18.7

Figure 4. Effect of hesperidin on body weight in the different experimental groups at the end of the experiment.

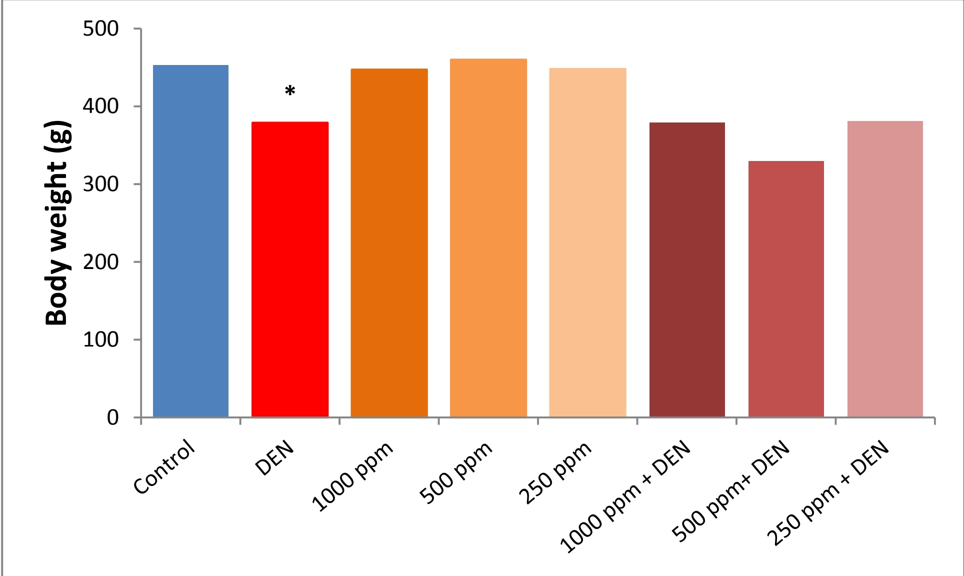
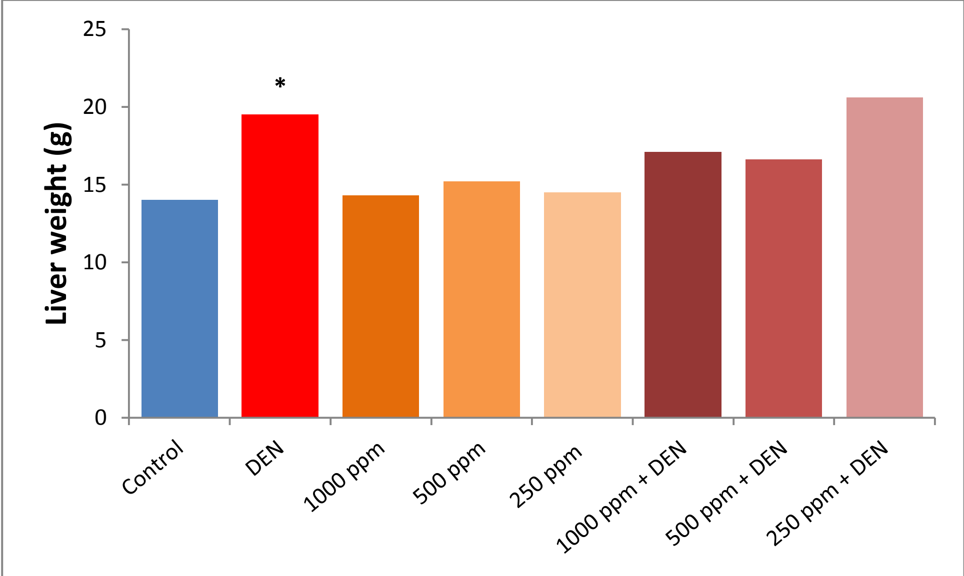


Figure 5. Effect of hesperidin on the liver weight in the different experimental groups. * p<0.05.

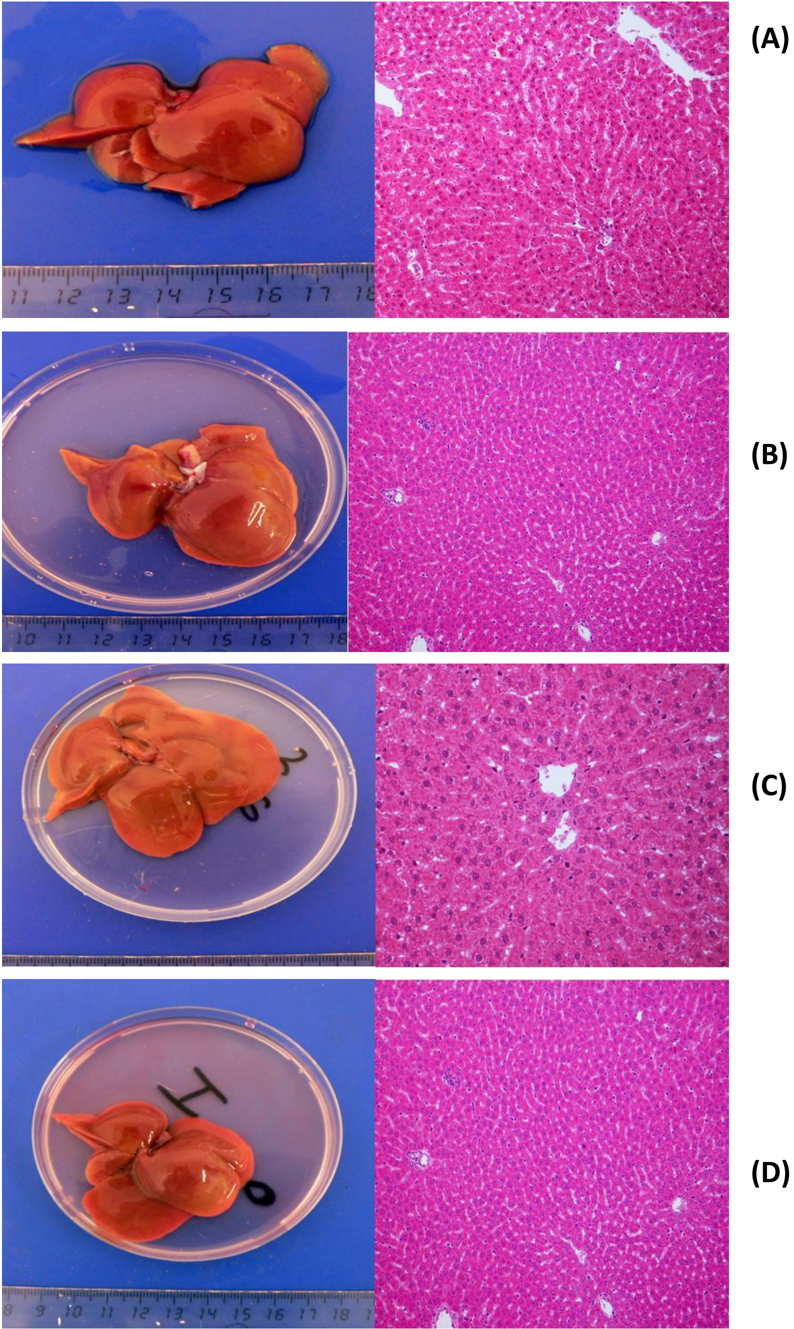


Effect of hesperidin on liver nodules growth.

No macroscopic liver nodules were observed in livers from negative control group as well as in hesperidin control groups (Groups 1, 2 and 3) These livers showed the typical smooth surface without irregularities (**Figure 6 and** 8). However, macroscopic liver nodules were found in DEN-exposed group (Figure 7 (A)). These nodules of various sizes were white to gray-white color and were within liver parenchyma or projected from the surface. About 95% of the nodules measured between 1-3 mm.

Hesperidin at a dose of 1000 ppm combined with DEN exhibited a significant decrease of liver nodule growth as compared with DEN group (**Figure 7 (B)** and **8** and **Table 2**). DEN + 500 or 250 ppm of hesperidin also decreased the liver nodule growth at the end of the experiment but it was not reached statistical significance (Figure 7 (C) and (D), 8 and Table 2).

Figure 6. Macroscopic and histopathological examination of the livers at the end of the study. A: Representative liver from Negative control; B: 1000 ppm hesperidin treatment; C: 500 ppm hesperidin treatment; D:250 ppm hesperidin treatment . H&E. 10X.



(A)

(B)

(C)

(D)

Figure 7. Macroscopic and histopathological examination of liver at the end of the study. Representative liver from DEN group (A), 1000 ppm + DEN group (B), 500 ppm + DEN group (C) and 250 ppm + DEN group (D).

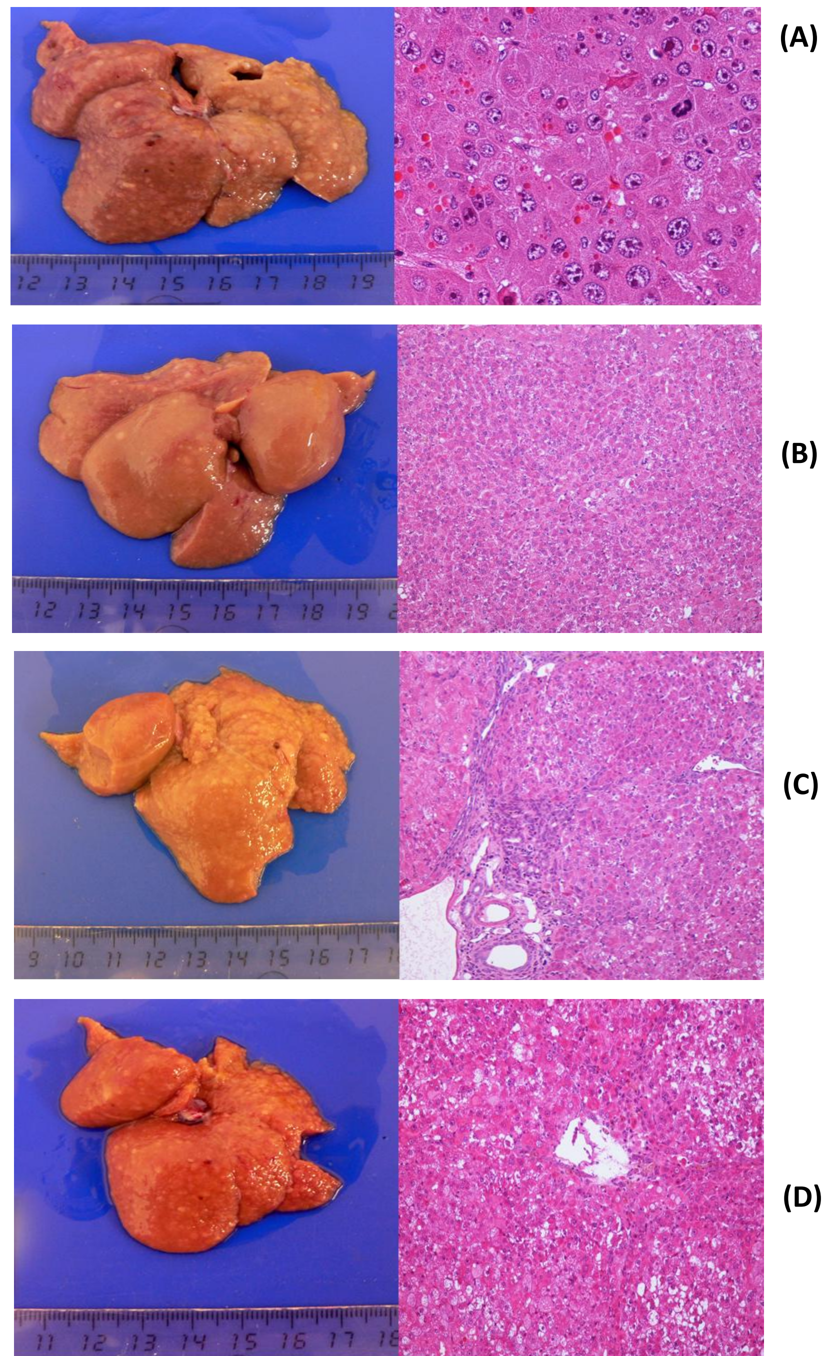
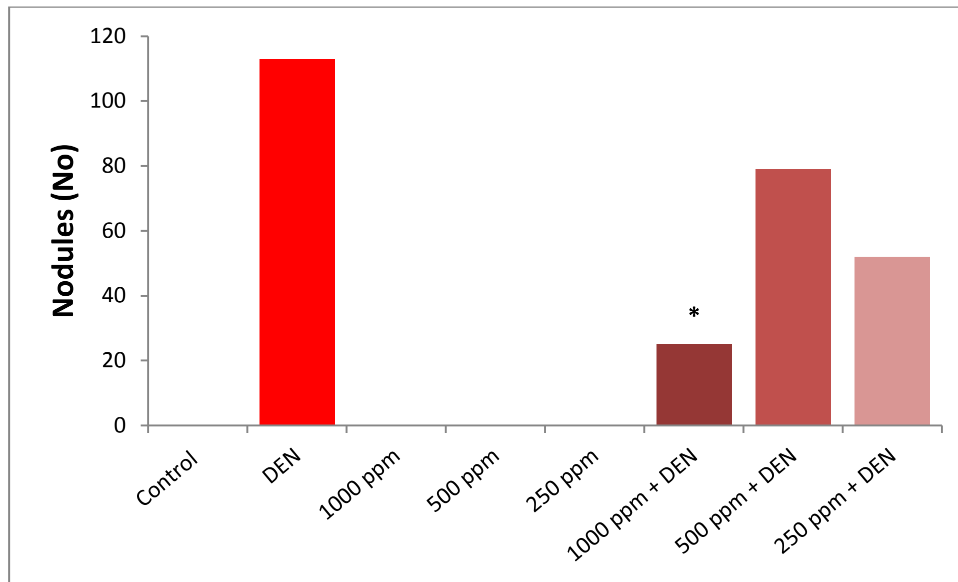


Figure 8. Effect of hesperidin on liver nodule growht in the different experimental groups at the end of the study.



<u>Effect of hesperidin on hepatic histology</u>

In the histopathological analysis of the liver, negative control group and hesperidin control groups (Groups 1, 2 and 3) showed the typical lobular architecture of polyhedral hepatocytes with granular cytoplasm and small uniform nuclei arranged in cords and with a mitotic index of 0-1 in 10 high-power fields (**Figure 6**). In contrast, livers from animals exposed to DEN, presented complete loss of normal architecture, with irregular shape of hepatocytes and enlarged and hyperchromatic nuclei and with a mitotic index of 8-9 in 10 high-power fields (**Figure 7**). Several hepatic lesions were observed in DEN treated group: foci of cellular alterations, bile duct hyperplasia, oval cell hyperplasia, regenerative hyperplasia, cholectasis, hepatic necrosis, focal fatty change and inflammation. The main found lesion was foci of cellular alteration composed of usually enlarged

polygonal hepatocytes with acidophilic staining cytoplasm from the surrounding normal parenchyma

The treatment of 1000 ppm of hesperidin combined with DEN exhibited the most remarkable effect on the liver histopathology. In this group, a striking improvement in the hepatocellular architecture was observed with more regular and less altered hepatocytes as compared to DEN group (Figure 7 (B)). Nevertheless, no significant changes in mitotic index were observed.

DISCUSSION

Although the inhibition of carcinogenesis by some dietary polyphenols has been extensively studied, the molecular mechanisms of action as well as histopathological aspects in a *in vivo* setting are less well understood.

One of the molecular mechanism what seems to be affected by dietary polyphenols is DNA methylation. Genome-wide DNA hypomethylation has been associated to epigenetic mechanism in malignancies and may play a crucial role in carcinogenesis (Chalitchagorn et al., 2004).In this study we show for the first time that different doses (0.78, 3, 6, 12.5 and 25 mM) of hesperidin can modulate DNA methylation pattern at different levels in the repetitive sequences LINE1, ALU, SAT-α, and SAT-2 in HL60 cells.

The selected doses of hesperidin used in the *in vitro* evaluation of the methylation status were selected taken into consideration both the presence of this polyphenol in the human daily intake (250 mL orange juice) and the results obtained in the present cytotoxic control assay. These

results are in accordance with previous *in vitro* results obtained with the same cell line being the IC_{50} similar (Fernández-Bedmar et al., 2011).

DNA methylation changes may affect to the repetitive DNA sequences that are comparatively rich in CpG dinucleotides (Wilson et al., 2007). Repetitive sequences represent about the 50 % of the human genome (Lander et al., 2001). For this reason they are promising targets for chemotherapy. LINE-1 repetitive transposable elements are the most important repetitive element in the human genome (Pandey et al., 2010) with 500,000 copies that represent 17% of the human genome mass (Prak and Haoudi, 2006). Alu repeats are present in 1,179,211 copies in the genome which together account for nearly 10.8% of the of human genome (Grover et al., 2004). Satellite α (Sat-α) repeats are DNA sequences of 170 bp and represent the main DNA component of every human centromere (Lee et al., 1997). Satellite 2 (Sat-2) DNA sequences are the most conspicuous in the long juxtacentromeric heterochromatin region of chromosomes representing the 2% of human genome (Tagarro et al., 1994).

Studies have been carried out using diet components as possible modulator agents of DNA methylation in cancer cells. Epigenetic therapy against harmful effects of diet components could be a potential tool in chemotherapy because epigenetic defects are reversible in contrast to genetic defects (Issa, 2002; Miyamoto and Ushijima, 2005). Diets components can act in a dual way by increasing or decreasing the methylation levels depending on the specific gene promoter or the genomic region. Curcumin did not decrease the LINE-1 methylation but induced demethylation of specific CpG loci in colon cancer cell lines

(HCT116, HT29 and RKO) (Link et al., 2013). Similarly, neither green tea poliphenols unmethylated LINE-1 sequences in prostate cancer cell line (Pandey et al., 2010). Contrarily, apple polyphenols exerted demethylating activity in tumour suppressor genes (hMLH1, p^{14ARF}, and p^{16INK4}) through the inhibition of DNA methyltransferases in colorectal cancer cells (Fini et al., 2007). Genistein, the major isoflavone present in soy beans exhibited a dose-dependent inhibition of DNA methyltransferase activity in KYSE510 cells (Fang et al., 2005). Lycopene induced the demethylation of the *GSTP1* tumor suppressor gene in MDA-MB-468 breast cancer cells (King-Batoon et al., 2008). Nevertheless Epigallocatechin-3-gallate, the major polyphenol present in green tea did not show any notable decrease in methylation of Alu sequences in urinary bladder transitional cell carcinoma, prostate adenocarcinoma and colorectal adenocarcinoma (T24, PC3 and HT29 respectively) (Chuang et al., 2005).

5-Aza-2´-deoxycytidine (decitabine) has been approved by the FDA as molecule for the treatment of myelodysplastic sindromes (Mack, 2006) and is a synthetic demethylating agent that has been showed to reactivate tumour suppressor genes silenced by promotor DNA methylation(Jones and Baylin, 2002; Jones and Laird, 1999; Momparler and Bovenzi, 2000). Lemaire et al. (2008) showed that this molecule was able to demethylate LINE 1 promotor in human and murine cancer leukemia cell lines. Furthermore, decitabine (commercial name of 5-az-2´-DC) decreased DNA methylation of Alu and LINE repetitive element by 9% and 16 % respectively in patients myeloid leukemia (Yang et al., 2006). Hipermethylation of LINE-1 promoter is considered as a profiling mark of

aberrantly hypermethylated tumor suppressor genes (TSG) in human cultured melanoma cell lines (Tellez et al., 2009).

Interestingly, the demethylating effects of hesperidin found in our study on the LINE-1 and Alu promoter of HL60 leukaemia cells was similar to the obtained by those authors with the synthetic and toxic 5-aza-2dC demethylating agent. Previous studies using hesperetin and naringenin (significant phenols contained in oranges) inhibited the DNA metiltransferase activity at 20 and 50 µmol/L in a dose-depend manner in esophageal cancer cell line (KYSE510) (Fang et al., 2007). This suggests that it is possible to achieve a chemopreventive effect against DNA methylation mechanism with polyphenols such as hesperidin.

On the other hand, in order to confirm the benefits effects of this polyphenol,in an *in vivo* setting, a rat model of DEN-induced hepatocellular carcinoma has also been performed. DEN treatment significantly decreased body weights, increased the liver weight and nodule growth and induced a complete loss of normal liver architecture with altered hepatocytes and numeours preneoplastic lesions at the end of the experiment. These altered hepatocytes were aggregated in clusters resulting in the appearance of prominent eosinophilic alteration focus with less than 3 cm in diameter (>95%). These focuses are usually found in short-duration toxicity studies with rodent models, as the present study, following exposure of a hepatocarcinogen such as nitrosamines (Thoolen et al., 2010). All these lesions are expected since DEN is an environmental and dietary hepatocarcinogen which induces tumors very similar to human counterpart (Lee et al., 2004; Peto et al., 1991; Verna et al., 1996). Several

studies have reported that these liver DEN-induced nodules are precursors of hepatic cancer (Farber and Sarma, 1987; Williams, 1980). Thus, present results confirm this well-established animal model for the study of liver carcinogenesis as others have reported (Du et al., 2009). In addition, administration of hesperidin at the different doses did not affect to body and liver weight indicating that these doses were well tolerated by rats with adequate growth responsive effect during the experiment.

Dietary administration of 1000 ppm of hesperidin inhibited hepatocarcinogenesis as revealed by a significant reduction of liver nodule growth and a striking improvement of hepatocellular architecture as compared with controls. Thus, the liver architecture of this group seemed to be similar that of normal liver in control group with more regular morphology and few altered hepatic cell foci. Besides, the well-known cardioprotective and anti-inflamatory effects of hesperidin, anticarcinogenic effects have been attributed to this bioflavonoid Al-Jasabi and Abdullah (2013) demonstrated a protective effect of hesperidin in B(a)p-induced lung cancer since it attenuated alteration of serum biomarkers. Moreover, it has been demonstrated that the same doses used in this study, 1000 ppm of hesperidin, had chemopreventive effects in azoxymethane-induced rat colon carcinogenesis (Tanaka et al., 1997a).

The mechanisms which hesperidin improved the liver architecture remain unclear. Some authors have reported that hesperidin exerts antioxidant effects by several mechanisms including reduction of expression of intercellular adhesion molecule-1 (ICAM-1) (Kim et al., 2011), suppression of gene expression of several proinflamatory cytokines (TNF-alpha, IL-

1beta, IL-6) and increase of the expression of Nf-E2-related factor 2 (NRF2) a key regulator of the expression of enzymes such as glutathione S-transferase (GST) and quinine reductase (QR) with potent antioxidant activity (Cha et al., 2001; Elavarasan et al., 2012; Lee et al., 2011). Nevertheless, despite evidence of these antioxidant effects, more studies are necessary to clarify this matter. Finally, 500 ppm and 250pm of hesperidin did not seem be sufficiently effective to affect on the different analyzed parameters since no significant differences were observed.

Here we have demonstrated the induction of global demethylation in LINE-1 and ALU-M2 repetitive sequences by hesperidin on leukemia model cell. Demethylation in satellite sequences Sat-alfa and Sat 2 was not observed in hesperidin-treated cells, probably due to the crucial role of satellite sequences in cell survival. A promising perspective is displaying after the present work as we propose it as a molecule of choice in epigenetic therapies using foods as a nutraceutical. Synthetic molecules like decitabine could be substituted by natural origin molecules like hesperidin which has the advantage of its lack of genotoxicity, antigenotoxic activity and cytotoxic effect in cancer cell lines. Furthermore, the data described in this pilot experience showed that hesperidin is a promising candidate agent for hepatocelullar chemoprevention since its beneficial effects on DNA methylation and histopathological parameters in a *in vitro* and *in vivo* setting. Future studies are needed to investigate and analyze in detail the molecular and pathological mechanisms through which this bioflavonoid can exert their protective effects against cancer.

REFERENCES

Al-Jasabi, S., Abdullah, M., 2013. The role of antioxidant hesperidin in the attenuation of lung cancer caused by benzo [a] pyrene in Balb/c mice. World Appl. Sci. J. 22, 1106-1110.

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Gene. Dev. 16, 6-21.

Bishayee, A., Dhir, N., 2009. Resveratrol-mediated chemoprevention of diethylnitrosamine-initiated hepatocarcinogenesis: inhibition of cell proliferation and induction of apoptosis. Chem.-Biol. Interact. 179, 131-144.

Bishayee, A., J Thoppil, R., Waghray, A., A Kruse, J., A Novotny, N., S Darvesh, A., 2012. Dietary phytochemicals in the chemoprevention and treatment of hepatocellular carcinoma: in vivo evidence, molecular targets, and clinical relevance. Curr. Cancer Drug Tar. 12, 1191-1232.

Cha, J.-Y., Cho, Y.-S., Kim, I., Anno, T., Rahman, S., Yanagita, T., 2001. Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats. Plant Foods Hum. Nutr. 56, 349-358.

Chalitchagorn, K., Shuangshoti, S., Hourpai, N., Kongruttanachok, N., Tangkijvanich, P., Thong-ngam, D., Voravud, N., Sriuranpong, V., Mutirangura, A., 2004. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene 23, 8841-8846.

Chiba, H., Uehara, M., Wu, J., Wang, X., Masuyama, R., Suzuki, K., Kanazawa, K., Ishimi, Y., 2003. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. J. Nutr. 133, 1892-1897.

Chuang, J.C., Yoo, C.B., Kwan, J.M., Li, T.W., Liang, G., Yang, A.S., Jones, P.A., 2005. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. Mol. Cancer Ther. 4, 1515-1520.

da Silva, J., 2005. Influence of orange juice over the genotoxicity induced by alkylating agents: an in vivo analysis. Mutagenesis 20, 279-283.

Davis, C.D., Uthus, E.O., 2004. DNA methylation, cancer susceptibility, and nutrient interactions. Exp. Biol. Med. 229, 988-995.

Du, Y.-p., Peng, J.-s., Sun, A., Tang, Z.-h., Ling, W.-h., Zhu, H.-l., 2009. Assessment of the effect of betaine on p16 and c-myc DNA methylation and mRNA expression in a chemical induced rat liver cancer model. BMC Cancer 9, 261.

Elavarasan, J., Velusamy, P., Ganesan, T., Ramakrishnan, S.K., Rajasekaran, D., Periandavan, K., 2012. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. J. Pharm. Pharmacol. 64, 1472-1482.

Fang, M., Chen, D., Yang, C.S., 2007. Dietary polyphenols may affect DNA methylation. J. Nutr. 137, 223S-228S.

Fang, M.Z., Chen, D., Sun, Y., Jin, Z., Christman, J.K., Yang, C.S., 2005. Reversal of hypermethylation and reactivation of p16INK4a, RARβ, and MGMT genes by genistein and other isoflavones from soy. Clin. Cancer Res. 11, 7033-7041.

Farber, E., Sarma, D., 1987. Hepatocarcinogenesis: a dynamic cellular perspective. Lab. Invest. 56, 4-22.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Fini, L., Selgrad, M., Fogliano, V., Graziani, G., Romano, M., Hotchkiss, E., Daoud, Y.A., Edward, B., Boland, C.R., Ricciardiello, L., 2007. Annurca apple polyphenols have potent demethylating activity and can reactivate silenced tumor suppressor genes in colorectal cancer cells. J. Nutr. 137, 2622-2628.

Forner, A., Bruix, J., 2012. Biomarkers for early diagnosis of hepatocellular carcinoma. Lancet Oncol. 13, 750-751.

Garg, A., Garg, S., Zaneveld, L., Singla, A., 2001. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. Phytother. Res. 15, 655-669.

Gattuso, G., Barreca, D., Gargiulli, C., Leuzzi, U., Caristi, C., 2007. Flavonoid composition of citrus juices. Molecules 12, 1641-1673.

González-Molina, E., Domínguez-Perles, R., Moreno, D., García-Viguera, C., 2010. Natural bioactive compounds of Citrus limon for food and health. J. Pharmaceut. Biomed. 51, 327-345.

Grover, D., Mukerji, M., Bhatnagar, P., Kannan, K., Brahmachari, S.K., 2004. Alu repeat analysis in the complete human genome: trends and variations with respect to genomic composition. Bioinformatics 20, 813-817.

Issa, J.-P., 2002. Epigenetic variation and human disease. J. Nutr. 132, 2388S-2392S.

Issa, J.-P.J., Kantarjian, H.M., 2009. Targeting DNA methylation. Clin. Cancer Res. 15, 3938-3946.

Jones, P.A., Baylin, S.B., 2002. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. 3, 415-428.

Jones, P.A., Laird, P.W., 1999. Cancer-epigenetics comes of age. Nat. Genet. 21, 163-167.

Kelloff, G.J., Boone, C.W., Crowell, J.A., Steele, V.E., Lubet, R., Sigman, C.C., 1994. Chemopreventive drug development: perspectives and progress. Cancer Epidem. Biomar. 3, 85-98.

Kim, S.-W., Kim, C.E., Kim, M.H., 2011. Flavonoids inhibit high glucose-induced up-regulation of ICAM-1 via the p38 MAPK pathway in human vein endothelial cells. Biochem. Bioph. Res. Co. 415, 602-607.

King-Batoon, A., Leszczynska, J.M., Klein, C.B., 2008. Modulation of gene methylation by genistein or lycopene in breast cancer cells. Environ. Mol. Mutagen. 49, 36-45.

Klimczak, I., Małecka, M., Szlachta, M., Gliszczyńska-Świgło, A., 2007. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. J. Food Compos. Anal. 20, 313-322.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., 2001. Initial sequencing and analysis of the human genome. Nature 409, 860-921.

Lee, C., Wevrick, R., Fisher, R., Ferguson-Smith, M., Lin, C., 1997. Human centromeric DNAs. Hum. Genet. 100, 291-304.

Lee, J.-S., Chu, I.-S., Mikaelyan, A., Calvisi, D.F., Heo, J., Reddy, J.K., Thorgeirsson, S.S., 2004. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat. Genet. 36, 1306-1311.

Lee, Y.-R., Jung, J.-H., Kim, H.-S., 2011. Hesperidin partially restores impaired immune and nutritional function in irradiated mice. J. Med. Food 14, 475-482.

Lemaire, M., Chabot, G.G., Raynal, N.J., Momparler, L.F., Hurtubise, A., Bernstein, M.L., Momparler, R.L., 2008. Importance of dose-schedule of 5-aza-2'-deoxycytidine for epigenetic therapy of cancer. BMC Cancer 8, 128.

Li, Y., Tollefsbol, T.O., 2010. Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. Curr. Med. Chem. 17, 2141.

Link, A., Balaguer, F., Goel, A., 2010. Cancer chemoprevention by dietary polyphenols: promising role for epigenetics. Biochem. Pharmacol. 80, 1771-1792.

Link, A., Balaguer, F., Shen, Y., Lozano, J.J., Leung, H., Boland, C.R., Goel, A., 2013. Curcumin modulates DNA methylation in colorectal cancer cells. Plos One 8, e57709.

Mack, G.S., 2006. Epigenetic cancer therapy makes headway. J. Natl. Cancer Inst. 98, 1443-1444.

Middleton Jr, E., Kandaswami, C., 1994. Potential health-promoting properties of citrus flavonoids. Food Technol.-Chicago.

Miyake, Y., Yamamoto, K., Tsujihara, N., Osawa, T., 1998. Protective effects of lemon flavonoids on oxidative stress in diabetic rats. Lipids 33, 689-695.

Miyamoto, K., Ushijima, T., 2005. Diagnostic and therapeutic applications of epigenetics. Jpn. J. Clin. Oncol. 35, 293-301.

Momparler, R.L., Bovenzi, V., 2000. DNA methylation and cancer. J. Cell. Physiol. 183, 145-154.

Pandey, M., Shukla, S., Gupta, S., 2010. Promoter demethylation and chromatin remodeling by green tea polyphenols leads to re-expression of GSTP1 in human prostate cancer cells. Int. J. Cancer 126, 2520-2533.

Pang, R., Tse, E., Poon, R.T., 2006. Molecular pathways in hepatocellular carcinoma. Cancer Lett. 240, 157-169.

Peto, R., Gray, R., Brantom, P., Grasso, P., 1991. Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. Cancer Res. 51, 6452-6469.

Prak, N.L., Haoudi, A., 2006. LINE-1 retrotransposition: impact on genome stability and diversity and human disease. Biomed. Res. Int. 2006.

Singh, S., Singh, P.P., Roberts, L.R., Sanchez, W., 2014. Chemopreventive strategies in hepatocellular carcinoma. Nat. Rev. Gastro. Hepat. 11, 45-54.

So, F.V., Guthrie, N., Chambers, A.F., Moussa, M., Carroll, K.K., 1996. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices.

Tagarro, I., Fernández-Peralta, A.M., González-Aguilera, J.J., 1994. Chromosomal localization of human satellites 2 and 3 by a FISH method using oligonucleotides as probes. Hum. Genet. 93, 383-388.

Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Ogawa, H., 1997a. Chemoprevention of

azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18, 957-965.

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara, A., Sumida, T., Fukutani, K., Tanaka, T., Ogawa, H., 1997b. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. Cancer Res. 57, 246-252.

Tanaka, T., Sugie, S., 2007. Inhibition of colon carcinogenesis by dietary non-nutritive compounds. J. Toxicol. Pathol. 20, 215-235.

Tellez, C.S., Shen, L., Estécio, M.R., Jelinek, J., Gershenwald, J.E., Issa, J.-P.J., 2009. CpG island methylation profiling in human melanoma cell lines. Melanoma Res. 19, 146-155.

Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Küttler, K., Deschl, U., 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. Toxicol. Pathol. 38, 5S-81S.

Vallejo, F., Larrosa, M., Escudero, E., Zafrilla, M.P., Cerdá, B., Boza, J., García-Conesa, M.T., Espín, J.C., Tomás-Barberán, F.A., 2010. Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. J. Agr. Food Chem. 58, 6516-6524.

Verna, L., Whysner, J., Williams, G.M., 1996. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol. Therapeut. 71, 57-81.

Vinson, J.A., Liang, X., Proch, J., Hontz, B.A., Dancel, J., Sandone, N., 2002. Polyphenol antioxidants in citrus juices: in vitro and in vivo studies relevant to heart disease, Flavonoids in Cell Function. Springer, pp. 113-122.

Weisenberger, D.J., Campan, M., Long, T.I., Kim, M., Woods, C., Fiala, E., Ehrlich, M., Laird, P.W., 2005. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res. 33, 6823-6836.

Wilson, A.S., Power, B.E., Molloy, P.L., 2007. DNA hypomethylation and human diseases. BBA-Rev. Cancer 1775, 138-162.

Williams, G.M., 1980. The pathogenesis of rat liver cancer caused by chemical carcinogens. BBA-Rev. Cancer 605, 167-189.

Yang, A.S., Doshi, K.D., Choi, S.-W., Mason, J.B., Mannari, R.K., Gharybian, V., Luna, R., Rashid, A., Shen, L., Estecio, M.R., 2006. DNA methylation changes after 5-aza-2'-deoxycytidine therapy in patients with leukemia. Cancer Res. 66, 5495-5503.

DISCUSIÓN GENERAL

El mercado global de nutracéuticos y alimentos funcionales se ha convertido en una industria multimillonaria con una tasa promedio de crecimiento constante del 9,6% por año (Keservani et al., 2010), siendo Japón el segundo mayor mercado de nutracéuticos después de Estados Unidos.

La búsqueda de nutracéuticos o alimentos funcionales no es fácil ni lineal puesto que no solo hay que estudiarlos desde un punto de vista. En este sentido, es necesario estudiarlos desde una perspectiva múltiple, es decir, a nivel individual, celular y molecular, con el objetivo de que su aplicación se traduzca en una mejor y más larga vida.

Estudios epidemiológicos apoyan el seguimiento de la dieta mediterránea como ejemplo de dieta saludable ya que ésta es un reservorio de sustancias que podrían ser utilizadas en la prevención y tratamiento de enfermedades degenerativas (Estruch et al., 2013; La Vecchia, 2004; Trichopoulou and Vasilopoulou, 2000). Sin embargo, hay escasos estudios clínicos basados en el estudio de los efectos saludables de estas sustancias. Entre ellos, un estudio llevado a cabo con 20 individuos, demostró que en individuos alimentados con una dieta de base mediterránea, disminuía la expresión de genes relacionados con la inflamación (*p65*, *MCP-1* y *TNF-α*) e incrementaba la expresión del gen anti-inflamatorio *IκBα* (Camargo et al., 2012).

En el presente trabajo se ha llevado a cabo un estudio integrativo multienfoque utilizando diversos ensayos que detectan diferentes actividades biológicas de componentes no procesados presentes en la

dieta mediterránea. La selección de los citados componentes está motivada en que, con la exclusión de los alimentos procesados presentes en la dieta mediterránea (aceite, vino y pan), ésta sería idéntica a la dieta de nuestros antepasados: la dieta paleolítica. Dicho estudio se ha llevado a cabo desde nivel poblacional pasando por el individual, celular y hasta llegar al nivel genético. Las muestras estudiadas han sido cuatro variedades de pimientos (*Capsicum anuum* and *Capsicum frutescens*), cebolla (*Allium cepa*), ajo (*Allium sativum*), tomate (*Solanum lycopersicum*), naranja (*Citrus sinensis*) y limón (*Citrus limon*), así como las moléculas más representativas incluidas en su matriz: capsaicina, capsantina y luteína en el caso del grupo de pimientos; dos organosulfurados representativos del género *Allium* (DPDS y DADS); licopeno como el carotenoide más abundante en el tomate y hesperidina y limoneno como moléculas presentes en la naranja y limón. A continuación se muestra una tabla con los resultados generales obtenidos en la presente tesis:

MUESTRA	SEGURIDAD	PROTECCIÓN (%)	QUIMIOPREVENCIÓN			LONG
			Citotoxicidad (CI_{50})	ADN	Comet	
PIMIENTOS						
Verde dulce	☺	☺ (64/73)	☺ (0.5 mg/mL)	☺	?	😐
Rojo dulce	☺	☺ (33/18)	☺ (0.6 mg/mL)	☺	?	☺
Verde picante	☺ ☹	☺☹ (64/-85)	☺ (1.5 mg/mL)	☺	?	☹
Rojo picante	☺	☺ (49/35)	☺(0.3 mg/mL)	☺	?	☹
Capsaicina	☺ ☹	☺ (33/62)	☺ (78 µM)	☺	?	☹
Capsantina	☺	☺ (49/28)	☺ (7 µM)	☹	?	☹
Luteína	☺	☺ (74/64)	😐	☹	?	☺
BULBOS						
Cebolla	☺	☺ (33/70)	😐	☹	☺	☹
Ajo	☺	☺ (19/59)	☺ (0.003 mg/mL)	☺	☺	☹
DPDS	☺	☺ (32/46)	☺ (0.25 mM)	☹	☺	☹
DADS	☺	☺ (40/95)	☺ (0.06 mM)	☺	☺	☹
TOMATE						
Tomate	☺	☺ (45/55)	☺ (0.75 mg/mL)	☹	☺	☹
Licopeno	☺	☺ (16/16)	😐	☹	☺	☹
CÍTRICOS						
Naranja	☺	☺ (44/50)	☺ (4.4 %)	☹	?	☺☹
Limón	☺ ☹	☺ (22/11)	☺ (1.4 %)	☺	?	☹
Hesperidina	☺	☺ (55/28)	☺ (14 mM)	☹	?	☺☹
Limoneno	☺ ☹	☺ (55/72)	☺ (0.2 mM)	☺	?	☺☹

☺: Efecto positivo; 😐: Sin efecto; ☹: efecto negativo; **?**: no ha podido llevarse a cabo el estudio; SEGURIDAD: ensayos de genotoxicidad; PROTECCIÓN: ensayos de antigenotoxicidad; ADN: ensayos de fragmentación; LONG: ensayos de longevidad.

La primera aproximación de esta tesis ha sido evaluar la seguridad de las muestras seleccionadas, tanto simples como complejas, desde el punto de vista genotoxicológico. Nuestros resultados confirman el uso seguro (no genotóxico) de todas las muestras seleccionadas excepto el pimiento verde picante, capsaicina, limón y limoneno a elevadas dosis a través del test de mutaciones y recombinaciones somáticas (SMART) de *Drosophila melanogaster*. Este ensayo ha sido seleccionado por su fiabilidad, facilidad y rapidez y porque permite el estudio de una amplia gama de sustancias tanto simples como complejas (Graf et al., 1984; Osaba et al., 1999; Villatoro-Pulido et al., 2009). Además se estudia por primera vez la actividad genotóxica del limón. Tanto en el caso del limón como en el de pimiento verde picante a las concentraciones más elevadas, hemos identificado, al menos parcialmente, las moléculas responsables de dicha actividad. Ésta podría deberse a sus moléculas más distintivas (capsaicina y limoneno respectivamente) puesto que las concentraciones de éstas se calcularon en base al contenido presente en sus correspondientes alimentos. Aunque hay pocos estudios que se centren en estudiar la actividad mutagénica de pimientos como mezclas complejas, todos ellos han mostrado que son seguros. Estos resultados están en concordancia general con los nuestros. Sin embargo el pimiento verde picante resultó no seguro a la concentración más elevada ensayada. Esta dosis equivaldría a 1-10 guindillas verdes encurtidas por día, producto que habitualmente se encuentra en tiendas de alimentación de nuestra geografía. Esta actividad biológica no deseable puede ser asociada al contenido en capsaicina, ya que ésta también resultó genotóxica a la concentración más alta ensayada, la cual es la correspondiente a la concentración más alta de pimiento

ensayada. La falta de seguridad en el uso del limoneno a altas concentraciones (50 veces la dosis establecida de zumo diaria) que han arrojado nuestros ensayos podría deberse a que esté actuando como un agente pro-oxidante y por lo tanto, cause estrés oxidativo y genere especies reactivas de oxigeno (Bakkali et al., 2008). Sin embargo el Programa de Toxicología Nacional en el año 2008 obtuvo todo lo contrario, aunque los ensayos se realizaron utilizando diferentes estirpes de *Salmonella typhimurium* que es un organismo procariota (Program, 1990). El test SMART además nos permite comprobar si las sustancias estudiadas (simples y complejas) son antigenotóxicas co-tratando las larvas de *Drosophila* con dichas sustancias conjuntamente con genotoxinas tales como el peróxido de hidrógeno, metil metasulfonato o etil carbamato (El Hamss et al., 2003; Fernández-Bedmar et al., 2011). Nuestros resultados han mostrado que todo el grupo seleccionado de pimientos (excepto el verde picante), el grupo de bulbos, tomate y cítricos modulan eliminando el efecto genotóxico causado por la genotoxina modelo peróxido de hidrógeno en porcentajes elevados. Hemos comprobado así mismo el efecto antigenotóxico de las moléculas más distintivas presentes en estos vegetales. De modo que, tanto muestras simples como complejas muestran distintos porcentajes de inhibición del daño genético causado por el peróxido de hidrógeno, variando entre el 16 y el 95%. Caso muy curioso el del pimiento verde picante, puesto que a pesar del efecto antigenotóxico de la capsaicina (molécula responsable de la pungencia de estos vegetales), éste no es capaz de inhibir la actividad genotóxica generada por el peróxido de hidrógeno, sino todo lo contrario. Por lo tanto, en la matriz del pimiento verde picante debe de tenerse en cuenta también

la existencia de otras moléculas que actúen negativa y sinérgicamente con el peróxido de hidrógeno. En general nuestros resultados apoyan los efectos beneficiosos de la dieta mediterránea. Tales beneficios se deben al contenido en fenoles y otras moléculas que constituyen cada uno de sus componentes y que son capaces de reducir el efecto genotóxico tanto de agentes endógenos como exógenos (Ortega, 2006; Saura-Calixto and Goñi, 2006).

En la presente tesis se muestran por vez primera los efectos que tiene una suplementación dietética, no exclusivamente calórica, sobre la longevidad usando diferentes variedades de pimiento, capsaicina, capsantina, tomate, cebolla, DADS, DPDS, naranja, limón, hesperidina y limoneno. Uno de los beneficios que confiere la dieta mediterránea es la calidad de vida asociada a la longevidad. Sin embargo, entre nuestros resultados de longevidad únicamente han mostrado efectos beneficiosos sobre ella el pimiento rojo dulce, naranja, hesperidina y limoneno. Debido a que la capsantina (carotenoide mayoritario en pimientos rojos) produjo un efecto negativo sobre la longevidad de *Drosophila melanogaster* no podemos atribuir a este carotenoide el efecto positivo del pimiento rojo dulce sobre la longevidad. Por lo tanto, otros componentes presentes en la matriz de los pimientos rojos dulces y ausentes en la matriz de los pimientos verdes deben estar actuando positivamente. En cuanto al efecto positivo de la naranja, hesperidina y limoneno hay que decir que solo fue notable a las concentraciones más bajas ensayadas. Con respecto al efecto negativo sobre la longevidad del resto de muestras ensayadas en la presente tesis,hay que tener en cuenta que no siempre una suplementación

dietética se traduce en un incremento de la longevidad (Tasset-Cuevas et al., 2013).

Otro aspecto importante a tener en cuenta en la detección de nutracéuticos o alimentos funcionales, es determinar si dichas sustancias tienen un efecto antiproliferativo en células cancerosas (efecto citotóxico). La línea celular HL60 es un modelo robusto para estudiar el efecto citotóxico de sustancias con potencial quimiopreventivo (Faujan et al., 2013). Para analizar esta actividad se utilizó un amplio rango de concentraciones de cada sustancia. En la presente tesis se muestra por vez primera el efecto antiproliferativo que tiene el pimiento fresco sobre células cancerígenas. Nuestros resultados muestran que las cuatro variedades de pimiento, ajo, tomate, naranja y limón así como capsaicina, capsantina, DADS, DPDS, hesperidina y limoneno exhiben diferentes actividades antiproliferativas frente a la línea tumoral HL60. De hecho, hay que destacar que el valor más bajo obtenido para la IC_{50} de entre todos los alimentos completos es la del ajo (0.003 mg/mL). Sin embargo, cebolla, luteína y licopeno no llegaron a alcanzar la IC_{50}.

Ensayos de citotoxicidad realizados con capsaicina en diferentes líneas cancerosas (HL60, SNU-1, SHSY-5Y, PC-3, LNC_aP, AGS, A172, SK-Hep-1) apoyan nuestros resultados (Gil and Kang, 2008; Ito et al., 2004; Jung et al., 2001; Kang et al., 2001; Kim et al., 1997; Lo et al., 2005; Mori et al., 2006; Richeux et al., 1999). Aunque no encontramos concordancia con los resultados obtenidos por Murakami et al. (2000) los cuales mostraron que la capsantina no presentaba un notable efecto antiproliferativo a 25µM, sin embargo, nosotros obtuvimos la IC_{50} a 7µM, una concentración

aproximadamente 4 veces más baja que la ensayada por estos autores. Diferentes extractos obtenidos a partir de tomate han mostrado tener efecto antiproliferativo en líneas cancerosas de estómago, hígado, mama y colon y además en basófilos de leucemia de ratas (Ferreres et al., 2010; Friedman et al., 2009), resultados que están en concordancia con los nuestros. Sin embargo, los resultados de citotoxicidad obtenidos por otros autores con licopeno son controvertidos, poniendo de manifiesto que el efecto antiproliferativo de esta molécula depende tanto del modelo tumoral como de la dosis ensayada (Salman et al., 2007; Teodoro et al., 2012). Observando nuestros resultados de citotoxicidad con bulbos y sus moléculas distintivas, podemos sugerir que éstas mimetizan el efecto citotóxico de la matriz vegetal donde se encuentran. Además los resultados obtenidos por otros autores concuerdan con los nuestros. Aunque los mecanismos moleculares que tienen lugar en este sentido con ajo y DADS son ampliamente estudiados, son escasos los estudiados con cebolla y DPDS. Diversos autores han mostrado la eficacia del zumo de naranja y limón para inhibir la proliferación de células cancerosas de leucemia, mama y colon entre otras (Camarda et al., 2007; Lim and Lim, 2006; Sun et al., 2002). Estudios *in vitro* llevados a cabo por Manthey and Guthrie (2002) con diferentes líneas tumorales (MDA-MB-435 ER-, MCF-7 ER+, DU-145, HT-29, DMS-114, SK-MEL5) demostraron que la hesperidina carecía de actividad antiproliferativa debido a la glicosilación de la mitad de esta molécula. Sin embargo estudios *in vivo* coinciden con nuestros resultados positivos para tal efecto. Estudios realizados con limoneno mostraron que este isoprenoide es citotóxico en células de leucemia HL60 (Tatman and Mo, 2002) sin embargo, los estudios *in vivo* son contradictorios: por una

parte inhibe la formación de tumores gástricos y de hígado y por otra induce tumores de riñón y vejiga (Lu et al., 2004; Parija and Das, 2003; Turner et al., 2001).

Una característica de las células tumorales es que tienen la capacidad de inactivar la apoptosis (muerte celular programada) y continuar creciendo exponencialmente. La apoptosis es un proceso clave en el desarrollo y promoción del cáncer y por ello la reactivación selectiva de este proceso en células cancerosas es uno de los mayores objetivos en el desarrollo de nuevas terapias contra el cáncer (Lowe and Lin, 2000).

La presente tesis muestra por vez primera el posible efecto apoptótico inducido por pimiento y tomate como mezclas complejas. Nuestros resultados muestran que todas las variedades de pimiento, ajo y limón así como capsaicina, DADS y limoneno tienen la capacidad de fragmentar el ADN (características del proceso apoptótico) de células HL60. Entre las variedades de pimientos, fueron las variedades picantes las que presentaban un patrón de fragmentos internucleosómicos proapoptóticos mucho más acusado que las variedades dulces. Esto podría explicarse por la presencia de capsaicina en las picantes, de hecho esta molécula también indujo un efecto pro-apoptótico de fragmentación del ADN. Numerosos trabajos han demostrado la actividad apoptótica de la capsaicina (Ito et al., 2004; Jun et al., 2007; Jung et al., 2001; Kim et al., 2004; Lee et al., 2000; Lo et al., 2005; Pramanik et al., 2011; Sánchez et al., 2007; Tsou et al., 2006; Wu et al., 2006). Tsou et al. (2006) demostraron que la inducción de apoptosis en HL60 se producía por activación de la caspasa 3. Hay escasos datos que muestren la actividad apoptótica de capsantina y luteína, y en

todos ellos se obtienen resultados positivos que se contradicen con los nuestros (Chew et al., 2002; MOLNAR et al., 2004; Zhang et al., 2011). Esto podría ser debido a que las concentraciones testadas de carotenoides por otros autores son mucho más elevadas que en nuestros ensayos o bien a que las líneas celulares empleadas y los ensayos son diferentes a los nuestros. Con respecto al grupo de los bulbos, es posible que la inducción de fragmentación del ADN se deba a su organosulfurado más característico, el DADS. De hecho se ha podido comprobar por otros autores que esta molécula induce apoptosis en HL60 por la expresión de la caspasa 3 (Kwon et al., 2002). Se ha podido comprobar que el limoneno incrementa las especies reactivas de oxígeno e induce apoptosis en células de cáncer de próstata y que aceites volátiles de lima activan la caspasa 3 e inducen fragmentación del ADN en células de cancer de colón (Patil et al., 2009; Rabi and Bishayee, 2009). A diferencia del limoneno, en el presente trabajo no se ha encontrado que la vía citotóxica de la hesperidina sea la proapoptótica por fragmentación internicleosómica del ADN.

Aunque la fragmentación del ADN es uno de los marcadores bioquímicos de apoptosis, éste no debería de tenerse en cuenta por si solo como criterio para asegurar la muerte celular programada (Cohen et al., 1992). Ya que aunque en algunos casos no se observe fragmentación del ADN, pueden existir otros marcadores que nos ayuden a profundizar en el estudio completo del proceso de apoptosis. En este sentido la observación de roturas de cadena simple a través del ensayo del cometa ha sido utilizado para detectar sustancias que sean capaces de causar daño en el ADN de células cancerosas (Balasenthil et al., 2002; Cardile et al., 2002). Es

la primera vez que se aplica este ensayo al ajo, cebolla y licopeno. En la mayoría de las sustancias ensayadas por nosotros se ha podido observar daño en el ADN de células HL60 (cebolla, ajo, tomate, DADS y licopeno). Mimetizando el licopeno el efecto del tomate. En la presente tesis se demuestra que el efecto anti-proliferativo del tomate y cebolla, así como las moléculas más representativas de ambos (licopeno y DPDS) en HL60 es por vía necrótica debido a que el valor del TM, aunque significativo, es inferior a 3. Estos resultados están en concordancia con los obtenidos en citotoxicidad y fragmentación del ADN, cosa que no ocurre con el ajo y DADS, en los que aunque se observe citotoxicidad y fragmentación del ADN, el valor de TM se corresponde con un proceso necrótico (TM menor que 3).

La presente Tesis muestra por primera vez los efectos de la hesperidina (molécula característica de la naranja) sobre la modulación de patrones de metilación en las secuencias repetitivas LINE1, ALU, SAT-α Y SAT-2 del ADN de células HL-60. Existen estudios que demuestran los efectos de componentes dietéticos sobre la modulación de marcas epigenéticas (Fini et al., 2007; King-Batoon et al., 2008; Link et al., 2013). Nuestros resultados han mostrado que dicha molécula hipometila las secuencias LINE1 y ALU. Previos estudios han mostrado que fenoles presentes en la naranja como la hesperetina y naringenina son capaces de inhibir la actividad metiltransferrasa de forma dosis-dependiente a 20-50 µmol/L en la linea tumoral KYSE510 (Fang et al., 2007). Es impotante señalar que nuestros resultados son similares a los efectos hipometiladores de la decitabina (molécula sintética y tóxica empleada en el tratamiento de síndromes

mielodisplásticos) en secuencias LINE1 y ALU en células HL60 (Lemaire et al., 2008) y en pacientes con leucemia(Yang et al., 2006). Esta similitud en los mecanismos moleculares de molélulas usadas en terapia frente al cáncer sugiere que los polifenoles naturales presentes en la dieta podrían sustituir o complementar la actividad de moléculas sintéticas utilizadas en quimioprevención a través de la terapia epigenética.

Con el fin de poder confirmar los efectos beneficiosos de este polifenol se llevó a cabo un estudio *in vivo* de carcinogénesis en ratas. Nuestros resultados mostraron que la DEN disminuye el peso de los animales tratados, incrementa el tamaño y el número de nódulos en el hígado e induce una pérdida de la arquitectura normal del hígado. Estos resultados son los esperados en de estudios de toxicidad a corto plazo con modelos roedores expuestos a hepatocarcinógenos como las nitrosaminas (Thoolen et al., 2010). Además, las lesiones son similares a las causadas por esta misma molécula en humanos (Lee et al., 2004; Peto et al., 1991; Verna et al., 1996) y los nódulos hallados son precursores de cáncer de hígado (Farber and Sarma, 1987; Williams, 1980). Por lo tanto ha sido bien establecido el modelo animal utilizado en el presente estudio como lo han mostrado también otros autores (Du et al., 2009). Adicionalmente, la hesperidina a las concentraciones ensayadas no afecta al peso del animal ni al hígado. Con respecto al efecto anticarcinogénico de la hesperidina, solamente la concentración más alta ensayada (1000 ppm) fue significativa en cuanto a la reducción del número de nódulos en el hígado y también se observó una mejora de la arquitectura hepática con respecto a su control. La hesperidina es un bioflavonoide al cual se le ha atribuido efectos

cardioprotectores y anti-inflamatorios (Al-Jasabi and Abdullah, 2013). Además la misma dosis empleada en este bioensayo(1000 ppm) ha demostrado tener efectos quimiopreventivos en cáncer de colon inducido por azoximetano en ratas (Tanaka et al., 1997). Sin embargo, los mecanismos a través de los cuales la hesperidina mejora la arquitectura hepática no están aún muy claros. Algunos autores han señalado que el efecto antioxidativo de esta molécula se originaría a través de mecanismos tales como la reducción de la expresión de la molécula-1 de adhesión celular (Kim et al., 2011), la inhibición de la expresión de genes proinflamatorios y el incremento de la expresión del factor NRF2 que es regulador de enzimas tales como GST y QT con una potente actividad antioxidante (Cha et al., 2001; Elavarasan et al., 2012; Lee et al., 2011). Sin embargo son necesarios más estudios para poder elucidar el posible mecanismo de acción de dicha molécula.

Según los resultados obtenidos en la presente tesis, y realizando una evaluación global, se ha podido comprobar que los alimentos estudiados son más saludables que los componentes más representativos presentes en ellos, al menos cuando se ensayan aislados. Por lo que en el efecto beneficioso de los indicadores de procesos degenerativos estudiados debe existir un sinergismo entre las diferentes moléculas presentes en la matriz del alimento completo. Al ser el alimento un reservorio de cientos de moléculas inmersas en su matriz, es imposible analizar la actividad de todos ellos con las herramientas actuales, para poder comprobar el efecto de cada uno de ellos. Por este motivo, a día de hoy la industria de la suplementación todavía no ha conseguido sorprendernos con un coctel de

antioxidantes que sea capaz de superar el efecto conseguido por una alimentación saludable y equilibrada. Además, muchas de las moléculas (biosaludables o no), mimetizan la actividad del alimento (saludable o no) en el que están contenidas aunque en menor medida.

REFERENCIAS

Al-Jasabi, S., Abdullah, M., 2013. The role of antioxidant hesperidin in the attenuation of lung cancer caused by benzo [a] pyrene in Balb/c mice. World Appl. Sci. J. 22, 1106-1110.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils–a review. Food Chem. Toxicol. 46, 446-475.

Balasenthil, S., Rao, K., Nagini, S., 2002. Garlic induces apoptosis during 7, 12-dimethylbenz [a] anthracene-induced hamster buccal pouch carcinogenesis. Oral Oncol. 38, 431-436.

Camarda, L., Di Stefano, V., Del Bosco, S.F., Schillaci, D., 2007. Antiproliferative activity of Citrus juices and HPLC evaluation of their flavonoid composition. Fitoterapia 78, 426-429.

Camargo, A., Delgado-Lista, J., Garcia-Rios, A., Cruz-Teno, C., Yubero-Serrano, E.M., Perez-Martinez, P., Gutierrez-Mariscal, F.M., Lora-Aguilar, P., Rodriguez-Cantalejo, F., Fuentes-Jimenez, F., 2012. Expression of proinflammatory, proatherogenic genes is reduced by the Mediterranean diet in elderly people. Brit. J. Nutr. 108, 500-508.

Cardile, V., Scifo, C., Russo, A., Falsaperla, M., Morgia, G., Motta, M., Renis, M., Imbriani, E., Silvestre, G., 2002. Involvement of HSP70 in resveratrol-induced apoptosis of human prostate cancer. Anticancer Res. 23, 4921-4926.

Cohen, G., Sun, X., Snowden, R., Dinsdale, D., Skilleter, D., 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem. J. 286, 331-334.

Cha, J.-Y., Cho, Y.-S., Kim, I., Anno, T., Rahman, S., Yanagita, T., 2001. Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats. Plant Foods Hum. Nutr. 56, 349-358.

Chew, B.P., Brown, C.M., Park, J.S., Mixter, P.F., 2002. Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis. Anticancer Res. 23, 3333-3339.

Du, Y.-p., Peng, J.-s., Sun, A., Tang, Z.-h., Ling, W.-h., Zhu, H.-l., 2009. Assessment of the effect of betaine on p16 and c-myc DNA methylation and mRNA expression in a chemical induced rat liver cancer model. BMC Cancer 9, 261.

El Hamss, R., Idaomar, M., Alonso-Moraga, A., Serrano, A.M., 2003. Antimutagenic properties of bell and black peppers. Food Chem. Toxicol. 41, 41-47.

Elavarasan, J., Velusamy, P., Ganesan, T., Ramakrishnan, S.K., Rajasekaran, D., Periandavan, K., 2012. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. J. Pharm. Pharmacol. 64, 1472-1482.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., Gómez-Gracia, E., Ruiz-Gutiérrez, V., Fiol, M., Lapetra, J., 2013. Primary prevention of cardiovascular disease with a Mediterranean diet. New Engl. J. Med. 368, 1279-1290.

Fang, M., Chen, D., Yang, C.S., 2007. Dietary polyphenols may affect DNA methylation. J. Nutr. 137, 223S-228S.

Farber, E., Sarma, D., 1987. Hepatocarcinogenesis: a dynamic cellular perspective. Lab. Invest. 56, 4-22.

Faujan, N.H., Alitheen, N., Yeap, S., Ali, A., Muhajir, A., Ahmad, F., 2013. Cytotoxic effect of betulinic acid and betulinic acid acetate isolated from Melaleuca cajuput on human myeloid leukemia (HL-60) cell line. Afr. J. Biot. 9, 6387-6396.

Fernández-Bedmar, Z., Anter, J., de La Cruz-Ares, S., Muñoz-Serrano, A., Alonso-Moraga, Á., Pérez-Guisado, J., 2011. Role of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity, and longevity in Drosophila. J. Toxicol. Env. Heal. A 74, 1052-1066.

Ferreres, F., Taveira, M., Pereira, D.M., Valentão, P.c., B. Andrade, P., 2010. Tomato (Lycopersicon esculentum) seeds: new flavonols and cytotoxic effect. J. Agr. Food Chem. 58, 2854-2861.

Fini, L., Selgrad, M., Fogliano, V., Graziani, G., Romano, M., Hotchkiss, E., Daoud, Y.A., Edward, B., Boland, C.R., Ricciardiello, L., 2007. Annurca apple polyphenols have potent demethylating activity and can reactivate silenced tumor suppressor genes in colorectal cancer cells. J. Nutr. 137, 2622-2628.

Friedman, M., Levin, C.E., Lee, S.-U., Kim, H.-J., Lee, I.-S., Byun, J.-O., Kozukue, N., 2009. Tomatine-containing green tomato extracts inhibit growth of human breast, colon, liver, and stomach cancer cells. J. Agr. Food Chem. 57, 5727-5733.

Gil, Y.-G., Kang, M.-K., 2008. Capsaicin induces apoptosis and terminal differentiation in human glioma A172 cells. Life Sci. 82, 997-1003.

Graf, U., Würgler, F., Katz, A., Frei, H., Juon, H., Hall, C., Kale, P., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environmental mutagenesis 6, 153-188.

Ito, K., Nakazato, T., Yamato, K., Miyakawa, Y., Yamada, T., Hozumi, N., Segawa, K., Ikeda, Y., Kizaki, M., 2004. Induction of Apoptosis in Leukemic Cells by Homovanillic Acid Derivative, Capsaicin, through Oxidative Stress Implication of Phosphorylation of p53 at Ser-15 Residue by Reactive Oxygen Species. Cancer Res. 64, 1071-1078.

Jun, H.-S., Park, T., Lee, C.K., Kang, M.K., Park, M.S., Kang, H.I., Surh, Y.-J., Kim, O.H., 2007. Capsaicin induced apoptosis of B16-F10 melanoma cells through down-regulation of Bcl-2. Food Chem. Toxicol. 45, 708-715.

Jung, M.-Y., Kang, H.-J., Moon, A., 2001. Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 downregulation and caspase-3 activation. Cancer Lett. 165, 139-145.

Kang, S.N., Chung, S.W., Kim, T.S., 2001. Capsaicin potentiates 1, 25-dihydoxyvitamin D 3-and all-trans retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Eur. J. Pharmacol. 420, 83-90.

Keservani, R.K., Kesharwani, R.K., Vyas, N., Jain, S., Raghuvanshi, R., Sharma, A.K., 2010. Nutraceutical and functional food as future food: A review. Der Pharmacia Lettre 2, 106-116.

Kim, C.-S., Park, W.-H., Park, J.-Y., Kang, J.-H., Kim, M.-O., Kawada, T., Yoo, H., Han, I.-S., Yu, R., 2004. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor γ in HT-29 human colon cancer cells. J. Med. Food 7, 267-273.

Kim, J.-D., Kim, J.-M., Pyo, J.-O., Kim, S.-Y., Kim, B.-S., Yu, R., Han, I.-S., 1997. Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. Cancer Lett. 120, 235-241.

Kim, S.-W., Kim, C.E., Kim, M.H., 2011. Flavonoids inhibit high glucose-induced up-regulation of ICAM-1 via the p38 MAPK pathway in human vein endothelial cells. Biochem. Bioph. Res. Co. 415, 602-607.

King-Batoon, A., Leszczynska, J.M., Klein, C.B., 2008. Modulation of gene methylation by genistein or lycopene in breast cancer cells. Environ. Mol. Mutagen. 49, 36-45.

Kwon, K.-B., Yoo, S.-J., Ryu, D.-G., Yang, J.-Y., Rho, H.-W., Kim, J.-S., Park, J.-W., Kim, H.-R., Park, B.-H., 2002. Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. Biochem. Pharmacol. 63, 41-47.

La Vecchia, C., 2004. Mediterranean diet and cancer. Public Health Nutr. 7, 965-968.

Lee, J.-S., Chu, I.-S., Mikaelyan, A., Calvisi, D.F., Heo, J., Reddy, J.K., Thorgeirsson, S.S., 2004. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat. Genet. 36, 1306-1311.

Lee, Y.-R., Jung, J.-H., Kim, H.-S., 2011. Hesperidin partially restores impaired immune and nutritional function in irradiated mice. J. Med. Food 14, 475-482.

Lee, Y.S., Nam, D.H., Kim, J.-A., 2000. Induction of apoptosis by capsaicin in A172 human glioblastoma cells. Cancer Lett. 161, 121-130.

Lemaire, M., Chabot, G.G., Raynal, N.J., Momparler, L.F., Hurtubise, A., Bernstein, M.L., Momparler, R.L., 2008. Importance of dose-schedule of 5-aza-2'-deoxycytidine for epigenetic therapy of cancer. BMC Cancer 8, 128.

Lim, S.-L., Lim, L.-Y., 2006. Effects of citrus fruit juices on cytotoxicity and drug transport pathways of Caco-2 cell monolayers. Int. J. Pharmaceut. 307, 42-50.

Link, A., Balaguer, F., Shen, Y., Lozano, J.J., Leung, H., Boland, C.R., Goel, A., 2013. Curcumin modulates DNA methylation in colorectal cancer cells. Plos One 8, e57709.

Lo, Y.-C., Yang, Y.-C., Wu, I.-C., Kuo, F.-C., Liu, C.-M., Wang, H.-W., Kuo, C.-H., Wu, J.-Y., Wu, D.-C., 2005. Capsaicin-induced cell death in a human gastric adenocarcinoma cell line. World J. Gastroentero. 11, 6254-6257.

Lowe, S.W., Lin, A.W., 2000. Apoptosis in cancer. Carcinogenesis 21, 485-495.

Lu, X.-G., Zhan, L.-B., Feng, B.-A., Qu, M.-Y., Yu, L.-H., Xie, J.-H., 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. World J. Gastroentero. 10, 2140-2144.

Manthey, J.A., Guthrie, N., 2002. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. J. Agr. Food Chem. 50, 5837-5843.

MOLNAR, J., GYÉMANT, N., MUCSI, I., MOLNAR, A., SZAB, M., KÖRTVÉLYESI, T., VARGA, A., MOLNAR, P., TOTH, G., 2004. Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. In Vivo 18, 237-244.

Mori, A., Lehmann, S., O'Kelly, J., Kumagai, T., Desmond, J.C., Pervan, M., McBride, W.H., Kizaki, M., Koeffler, H.P., 2006. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. Cancer Res. 66, 3222-3229.

Murakami, A., Nakashima, M., Koshiba, T., Maoka, T., Nishino, H., Yano, M., Sumida, T., Kim, O.K., Koshimizu, K., Ohigashi, H., 2000. Modifying effects of carotenoids on superoxide and nitric oxide generation from stimulated leukocytes. Cancer Lett. 149, 115-123.

Ortega, R., 2006. Importance of functional foods in the Mediterranean diet. Public Health Nutr. 9, 1136-1140.

Osaba, L., Aguirre, A., Alonso, A., Graf, U., 1999. Genotoxicity testing of six insecticides in two crosses of the Drosophila wing spot test. Mutat. Res. 439, 49-61.

Parija, T., Das, B.R., 2003. Involvement of YY1 and its correlation with c-myc in NDEA induced hepatocarcinogenesis, its prevention by d-limonene. Mol. Biol. Rep. 30, 41-46.

Patil, J.R., Jayaprakasha, G., Murthy, K.C., Tichy, S.E., Chetti, M.B., Patil, B.S., 2009. Apoptosis-mediated proliferation inhibition of human colon cancer cells by volatile principles of Citrus aurantifolia. Food Chem. 114, 1351-1358.

Peto, R., Gray, R., Brantom, P., Grasso, P., 1991. Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. Cancer Res. 51, 6452-6469.

Pramanik, K.C., Boreddy, S.R., Srivastava, S.K., 2011. Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells. Plos One 6, e20151.

Program, N.T., 1990. NTP Toxicology and Carcinogenesis Studies of d-Limonene (CAS No. 5989-27-5) in F344/N Rats and B6C3F1 Mice (Gavage Studies). National Toxicology Program technical report series 347, 1.

Rabi, T., Bishayee, A., 2009. d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J. Carcinogen. 8, 9.

Richeux, F., Cascante, M., Ennamany, R., Saboureau, D., Creppy, E.E., 1999. Cytotoxicity and genotoxicity of capsaicin in human neuroblastoma cells SHSY-5Y. Arch. Toxicol. 73, 403-409.

Salman, H., Bergman, M., Djaldetti, M., Bessler, H., 2007. Lycopene affects proliferation and apoptosis of four malignant cell lines. Biomed. Pharmacother. 61, 366-369.

Sánchez, A.M., Malagarie-Cazenave, S., Olea, N., Vara, D., Chiloeches, A., Díaz-Laviada, I., 2007. Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. Apoptosis 12, 2013-2024.

Saura-Calixto, F., Goñi, I., 2006. Antioxidant capacity of the Spanish Mediterranean diet. Food Chem. 94, 442-447.

Sun, J., Chu, Y.-F., Wu, X., Liu, R.H., 2002. Antioxidant and antiproliferative activities of common fruits. J. Agr. Food Chem. 50, 7449-7454.

Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Ogawa, H., 1997. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18, 957-965.

Tasset-Cuevas, I., Fernández-Bedmar, Z., Lozano-Baena, M.D., Campos-Sánchez, J., de Haro-Bailón, A., Muñoz-Serrano, A., Alonso-Moraga, Á., 2013. Protective effect of borage seed oil and gamma linolenic acid on DNA: in vivo and in vitro studies. Plos One 8, e56986.

Tatman, D., Mo, H., 2002. Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells. Cancer Lett. 175, 129-139.

Teodoro, A.J., Oliveira, F.L., Martins, N.B., Maia, G.d.A., Martucci, R.B., Borojevic, R., 2012. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. Cancer Cell. Int. 12, 36.

Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Küttler, K., Deschl, U., 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. Toxicol. Pathol. 38, 5S-81S.

Trichopoulou, A., Vasilopoulou, E., 2000. Mediterranean diet and longevity. Brit. J. Nutr. 84, S205-S209.

Tsou, M.-F., Lu, H.-F., Chen, S.-C., Wu, L.-T., Chen, Y.-S., Kuo, H.-M., Lin, S.-S., Chung, J.-G., 2006. Involvement of Bax, Bcl-2, Ca2+ and caspase-3 in capsaicin-induced apoptosis of human leukemia HL-60 cells. Anticancer Res. 26, 1965-1971.

Turner, S.D., Tinwell, H., Piegorsch, W., Schmezer, P., Ashby, J., 2001. The male rat carcinogens limonene and sodium saccharin are not mutagenic to male Big BlueTM rats. Mutagenesis 16, 329-332.

Verna, L., Whysner, J., Williams, G.M., 1996. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol. Therapeut. 71, 57-81.

Villatoro-Pulido, M., Font, R., De Haro-Bravo, M.I., Romero-Jiménez, M., Anter, J., Bailón, A.D.H., Alonso-Moraga, Á., Del Río-Celestino, M., 2009. Modulation of genotoxicity and cytotoxicity by radish grown in metal-contaminated soils. Mutagenesis 24, 51-57.

Williams, G.M., 1980. The pathogenesis of rat liver cancer caused by chemical carcinogens. BBA-Rev. Cancer 605, 167-189.

Wu, C.-C., Lin, J.-P., Yang, J.-S., Chou, S.-T., Chen, S.-C., Lin, Y.-T., Lin, H.-L., Chung, J.-G., 2006. Capsaicin induced cell cycle arrest and apoptosis in human esophagus epidermoid carcinoma CE 81T/VGH cells through the elevation of intracellular reactive oxygen species and Ca 2+ productions and caspase-3 activation. Mutat. Res.- Fund. Mol. M. 601, 71-82.

Yang, A.S., Doshi, K.D., Choi, S.-W., Mason, J.B., Mannari, R.K., Gharybian, V., Luna, R., Rashid, A., Shen, L., Estecio, M.R., 2006. DNA methylation changes after 5-aza-2′-deoxycytidine therapy in patients with leukemia. Cancer Res. 66, 5495-5503.

Zhang, X., Zhao, W.-e., Hu, L., Zhao, L., Huang, J., 2011. Carotenoids inhibit proliferation and regulate expression of peroxisome proliferators-activated receptor gamma (PPARγ) in K562 cancer cells. Arch. Biochem. Biophys 512, 96-106.

CONCLUSIONES GENERALES

En el estudio integrativo multienfoque recogido en la presente memoria de Tesis se ha llegado a las siguientes conclusiones sobre las actividades biológicas de pimiento, tomate, cebolla, ajo, naranja y limón así como las moléculas más representativas dentro de sus matrices como lo son capsaicina, capsantina, luteína, licopeno, DPDS, DADS, hesperidina y limoneno:

1. Ningún alimento ni molécula estudiada ha llegado a ser genotóxica en el ensayo de Mutaciones y Recombinaciones Somáticas de *Drosophila melanogaster*, es decir, son seguros, a excepción de las dosis altas de pimiento verde picante y limón así como capsaicina y limoneno.
2. Todos los alimentos y moléculas ensayadas han mostrado un efecto antigenotóxico o protector del daño oxidativo inducido por la genotoxina peróxido de hidrógeno aunque a diferentes niveles. El pimiento verde picante a altas dosis es el único alimento incapaz de proteger las células somáticas de *Drosophila* de dicho daño.
3. El pimiento rojo, la luteína y la naranja ha incrementado la longevidad de *Drosophila*, aunque las altas concentraciones ensayadas del resto de sustancias no han permitido encontrar el umbral de dosis correcto para determinar un incremento de longevidad por éstas.
4. Todos los alimentos y moléculas ensayadas han mostrado un efecto citotóxico en la línea promielocítica humana HL60. Luteína, cebolla y licopeno fueron las únicas sustancias incapaces de inhibir el crecimiento tumoral de dicha línea tumoral.

5. La causa del efecto citotóxico del pimiento, ajo, limón, capsaicina, DADS y limoneno puede ser la activación de la vía proapoptótica debido a la fragmentación observada en el ADN de las células tumorales.
6. La actividad clastogénica medida a nivel celular en la línea tumoral HL60 ha podido observarse en cebolla, tomate y ajo así como en DPDS, licopeno y DADS.
7. La molécula de elección hesperidina es capaz de ejercer desmetilación genómica en las secuencias repetitivas LINE-1 y AluM2.
8. La experiencia piloto de hepatocarcinogénesis inducida en ratas con dietil nitrosamina es muy prometedora ya que la hesperidina induce una inhibición de los nódulos del hígado originados por la dietil nitrosamina.
9. Como corolario, se muestra que no todos los componentes de la dieta mediterránea confieren el mismo nivel de protección del genoma de *Drosophila* ni son quimiopreventivos frente a células de leucemia. Además, el efecto de dosis es determinante en las actividades biológicas encontradas en las sustancias estudiadas.

GENERAL CONCLUSIONS

In the integrative and multiapproach study carried out in the present memory the following conclusions about the biological activities of pepper, tomato, onion, garlic, orange and lemon as well as the most representative molecules within their matrixes such as capsaicin, capsanthin, lutein, lycopene, DPDS, DADS, hesperidin and limonene have been reached:

1. No food or molecule studied showed to be genotoxic in the Somatic Mutation and Recombination test of *Drosophila melanogaster*. That is, they are safe, except the highest doses of green hot pepper and lemon, as well as capsaicin and limonene.
2. All tested foods and molecules showed different levels of antimutagenic effect or oxidative damage protection against hydrogen peroxide genotoxine. Green hot pepper at highest doses is the only food unable to protect the somatic cells of *Drosophila* from such damage.
3. Red pepper, lutein and orange have increased the *Drosophila* lifespan, although the highest assayed concentrations of other substances have not allowed us to find the correct dose threshold to determine an increase of lifespan by these.
4. All tested foods and molecules have shown a cytotoxic effect in the human promyelocytic HL60 line. Lutein, onion and lycopene were the only substances unable to inhibit the tumoural growth of this tumoural cell line.
5. The cause of the cytotoxic effect of the pepper, garlic, lemon, capsaicin, DADS and limonene can be the activation of the proapoptotic pathway due to the observed fragmentation in the tumoural cell DNA.

6. The clastogenic activity measured at cell level in the HL60 tumoural cell line has been observed in onion, garlic and tomato as well as in DPDS, DADS and lycopene.
7. Hesperidin is sable to exert a genomic demethylation in the LINE-1 and AluM2 repetitive sequences.
8. The pilot experience of diethylnitrosamine-induced hepatocarcinogenesis in rats is very promising as hesperidin induces a decrease of nodules originated by the carcinogen diethyl nitrosamine.
9. As corollary, it is shown that not all mediterranean diet components confer the same protection level to the *Drosophila* genome nor they are chemopreventive against leukemia cells. Furthermore, the dose-effect is critical for the biological activity found in the tested substances.

ANEXO: PUBLICACIÓN CAPÍTULO I

Journal of Toxicology and Environmental Health, Part A, 74:1052–1066, 2011
Copyright © Taylor & Francis Group, LLC
ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2011.582306

Taylor & Francis
Taylor & Francis Group

ROLE OF CITRUS JUICES AND DISTINCTIVE COMPONENTS IN THE MODULATION OF DEGENERATIVE PROCESSES: GENOTOXICITY, ANTIGENOTOXICITY, CYTOTOXICITY, AND LONGEVITY IN *DROSOPHILA*

Zahira Fernández-Bedmar, Jaouad Anter, Silvia de La Cruz-Ares, Andrés Muñoz-Serrano, Ángeles Alonso-Moraga, Joaquín Pérez-Guisado

Departamento de Genética, Universidad de Córdoba, Córdoba, Spain

It is well established that breakfast beverages contain high quantities of *Citrus* juices. The purpose of the present study was to assess the nutraceutical value of orange and lemon juices as well as two of their active compounds: hesperidin and limonene. Indicator assays were performed at three levels to evaluate different biological health promoter activities: (i) determination of the safety and DNA-damage protecting ability against free radicals by using the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*, (ii) study of the modulating role for life span in *Drosophila melanogaster*, and (iii) measurement of the cytotoxic activity against the human tumor cell line HL60. The highest concentrations assayed for lemon juice and limonene (50% v/v and 0.73 m*M*, respectively) showed genotoxic activity as evidenced from SMART. Orange and lemon juices as well as hesperidin and limonene exhibit antigenotoxic activity against hydrogen peroxide used as an oxidative genotoxin. Life-span experiments revealed that the lower concentrations of orange juice, hesperidin, and limonene exerted a positive influence on the life span of *Drosophila*. Finally all substances showed cytotoxic activity, with hesperidin being least active. Taking into account the safety, antigenotoxicity, longevity, and cytotoxicity data obtained in the different assays, orange juice may be a candidate as a nutraceutical food as it (1) is not genotoxic, (2) is able to protect DNA against free radicals, and (3) inhibits growth of tumor cells.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Inappropriate dietetic habits are estimated to be the cause of more than one-third of cancer deaths. Many of these cancers might be avoided with an increased consumption of fruits and vegetables (Smith-Warner et al. 2006). Plant-based foods provide the organism with high content in antioxidants that might help to protect cells from the biological damage produced by free radicals that trigger cancer development (Shi et al, 1998; Reddy et al. 2003). More precisely, fruit consumption has been associated with reduced risk of cancer of the upper digestive tract, stomach, and urinary tract (La Vecchia and Bossetti, 2006).

Orange juice (OJ) and lemon juice (LJ) contain a number of beneficial micronutrients, including phenols, vitamin C, minerals, dietetic fiber, essential oils, and carotenoids, that help to prevent degenerative processes such as diabetes, cardiovascular diseases, or certain types of cancer (A. A. Franke et al. 2005; González-Molina et al. 2010). The major flavonoid in sweet oranges and lemon is hesperidin (Gattuso et al. 2007; Garg et al. 2001), which is hydrolyzed by gut microflora into aglycone to form hesperetin (Vallejo et al. 2010). Hesperidin is used in treatment for hair fragility due to its ability to reduce the permeability of the vascular endothelium. This phenol exhibits antioxidative activity via antiradical and anti-lipoperoxidation activities (Tripoli et al. 2007). Hesperidin also exerts anti-

The authors express gratitude to the Consejeria de Innovacion, Ciencia y Empresa (Junta de Andalucia, Spain), for financial support.
Address correspondence to Ángeles Alonso-Moraga, Departamento de Genética, Universidad de Córdoba, Campus Rabanales, 145071 Córdoba, Spain. E-mail: ge1almoa@uco.es

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

inflammatory activity because it inhibits the LOX, COX, and phospholipase A enzymes (Benavente-García et al. 1997) and modulates glucose, cholesterol, and fatty acid metabolism (Jung et al. 2004; 2006). Hesperidin prevents bone mass loss (Chiba et al. 2003) and prevents chemically induced breast cancer (So et al. 1996), bladder cancer (Yang et al. 1997), and colon cancer (Tanaka et al. 1997a; 1997b; Miyagi et al. 2000) in animals.

The distinctive flavor component in OJ and LJ is limonene. This monocyclic terpene is the major component in the citrus essential oils (Crowell, 1999; González-Molina et al. 2010), used as flavor in cosmetics, beverages, foods, and gums. Although mutagenicity assays showed negative results in *Salmonella* (National Toxicology Program, 1990) and rats (Turner et al. 2001), it is considered a nongenotoxic carcinogen (Tennant and Ashby, 1991). Lu et al. (2004) suggested that limonene may be of interest in chemoprevention because it inhibits tumor growth and metastasis via apoptosis.

Fresh homemade citrus juices are one of the most popular fruit beverages as a member of so-called healthy breakfasts. Therefore, it was of interest to evaluate the nutraceutical potency of a chronically consumed food through the entire life of subjects Several testing protocols need to be established for a food to be considered a health promoter: (i) safety with respect to genetic damage; (ii) potential protective role of DNA integrity; (iii) influence on life-span extension as a complex biological trait; and (iv) specific cytotoxic activity against transformed cells as chemopreventive agent.

The somatic mutation and recombination test (SMART) has been used to detect mutagenic and recombinagenic activity in the clone expansion of imaginal discs of *Drosophila melanogaster* larvae. This wing spot test was found to be a versatile and reliable system to test genotoxicity and antigenotoxicity of single compounds as well as complex mixtures due to the capabilities of treated larvae to bioactivate metabolites (Graf et al. 1994; Anter et al. 2010). The ability of LJ, OJ, and two of their major components (hesperidin and limonene) to inhibit the mutagenicity induced by a model oxidative genotoxin such as hydrogen peroxide (H_2O_2) was examined. H_2O_2 produces oxidative damage to DNA by generating adducts, such as 8-hydroxyguanine, that exert an important role in the mutagenesis process with an increase of induced transitions (Shi et al. 1998; Lim and Lim 2006). Hydrogen peroxide induces also a deregulation of methylation patterns of oncogenes (Cerda and Weitzman 1997) and inhibition of DNA repair enzymes (Hu et al. 1995).

The expected health-promoting properties of LJ, OJ, and their distinctive compounds might extend the longevity in *Drosophila melanogaster*. The life span of this insect is relatively short and adults seem to show many of the cell senescence features seen in mammals (Fleming et al. 1992). For this reason the fruit fly has been extensively used in studies of physiological, pathological, and other processes involved in life expectancy, as well as to understand the relationships between food metabolism and ageing (Li et al. 2010). Average life-span data of *Drosophila melanogaster* vary widely and are dependent on the rearing conditions (Trotta et al. 2006; Li et al. 2008; Mockett and Sohal, 2006).

Cytotoxicity bioassays in vitro are also needed in the assessment of the chemopreventive effects of a substance as a fast, not expensive, and informative first step for screening. The human cell line HL60 provides a reliable model to study the cytotoxic effect of chemopreventive substances and the mechanisms underlying this potential activity (Villatoro-Pulido et al. 2009). Once the cytotoxic activity of a nutraceutic had been assayed, a visible test of DNA fragmentation was carried out in order to investigate whether the mechanism undergoing the cytotoxicity was mediated via apoptosis.

METHODS

Fruits and Single Compounds

Juices from two *Citrus* species and two single compounds were selected. Oranges (*Citrus sinensis* var. Valencia Late) and lemons (*Citrus*

limon var. Lunario) were obtained from a local market. Hesperidin and limonene were purchased from Sigma and Fluka (H5254 and 62118, respectively).

Preparation of the Samples

Fruits were washed with ethanol (70%) prior to extraction of the juice. Both OJ and LJ were prepared using a domestic manual squeezer. Fresh juices from 10 fruits were mixed, aliquoted, and stored at –80°C until utilization. In the case of cytotoxicity assays, juices were centrifuged for 1 min at 12,000 × g and the supernatant was stored at –80°C. Limonene was dissolved in ethanol.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Genotoxicity and Antigenotoxicity Assays (SMART)

Drosophila melanogaster strains. Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

- *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that produces multiple tricomas/cell (Yan et al. 2008).
- flr^3*/In (3LR) TM3, ri* p^p *sep* bx^{34e} e^s Bd^S, where the flr^3 (*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007). Lindsley and Zimm (1992) provide more detailed information on the rest of the genetic *Drosophila* markers.

Drosophila were maintained at 25°C, 80% humidity, with a homemade meal (1 L water, 0.5 g NaCl, 100 g yeast, 25 g sucrose, 12 g agar-agar, 5 ml propionic acid, 3.5 ml of a 0.2% sulfate streptomycin solution) with three changes per week.

Treatments. The genotoxicity assays were carried out following the method described by Graf et al. (1984). Briefly, trans-heterozygous larvae for *mwh* and flr^3 genes were obtained by crossing 200 optimally virgin females (4 d old) of flr^3 strain with 100 males of *mwh* strain. Four days after fertilization, females were allowed to lay eggs in fresh yeast medium for 8 h in order to obtain synchronized larvae. After 72 h, larvae were washed with distilled water and groups of 100 individuals were placed in different treatment vials where a chronic treatment was followed until pupation. Treatment vials contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 ml of different concentrations of the substance to be tested. Two concentrations of each citrus juice were assayed (0.75% v/v and 50% v/v) as well as hesperidin and limonene (0.0038 and 0.34 m*M*, 0.011 and 0.73 m*M*, respectively). Single compound concentrations correspond to the content level found in the fresh juices (Gattuso et al. 2007; Selli et al. 2004). The negative controls were prepared with medium and water and positive controls with medium and 0.15 *M* H_2O_2 (Sigma, H1009) as an oxidative genotoxicant (Anter et al. 2010).

The antigenotoxicity tests were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.15 *M* H_2O_2) and different concentrations of juices, hesperidin, or limonene. After emergence, adult flies were stored in 70% ethanol.

Mutations scoring. Forty trans-heterozygous marker wings (*mwh* flr^+/mwh^+ flr^3) of each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400× magnification. Similar numbers of male and female wings were mounted and wing hair mutations were scored from a total of 24,400 monotricoma wild-type cells per wing (Alonso-Moraga and Graf 1989). In the balancer-heterozygous genotypes (*mwh*/TM3, Bd^S), *mwh* spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homologue is a lethal event. To quantify the recombinagenic potency of the positive control, the frequency of *mwh* clones on the marker trans-hetorozygous wings (*mwh* single spots plus twin spots) was compared with the frequency of *mwh* spots

on the balancer trans-heterozygous wings. The difference in *mwh* clone frequency is a direct measure of the proportion of recombination (Frei et al. 1992). In the case of genotoxic results for single treatments, balancer wings (mwh/Bd^S) were also mounted in order to quantify the somatic recombinogenic activity (R) of the substance (Zordan et al. 1991) using the following formula:

$$R = (1 - mwh \text{ spots on the balancer wings}/mwh \text{ spots on the marker wings}) \times 100$$

Data evaluation and statistical analysis. Wing hair spots were grouped into three different categories: S, a small single spot corresponding to one or two cells exhibiting the *mwh* phenotype; L, a large single spot with three or more cells showing *mwh* or flr^3 phenotypes; or T, a twin spot corresponding to two juxtapositioning clones, one showing the *mwh* phenotype and other the flr^3 phenotype. Small and large spots are originated by somatic point mutation, chromosome aberration, and somatic recombination, while twin spots are produced exclusively by somatic recombination between the flr^3 locus and the centromere. The total number of spots was also determined.

A multiple-decision procedure was applied to determine whether a result is positive, inconclusive, or negative (Frei and Würgler, 1988, 1995). The frequencies of each type of mutant clone per wing were compared to the concurrent negative control and the significance was taken at the 5% level. All inconclusive and positive results were analyzed with the nonparametric *U*-test of Mann, Whitney, and Wilcoxon ($\alpha = \beta = .05$, one sided). In combined treatments the inhibition of mutagenic events for juices and single compounds was calculated for total spots as proposed by Abraham (1994) by means of the following formula:

$$\text{Inhibition} = (\text{genotoxin alone} - \text{sample plus genotoxin}) \times 100/\text{genotoxin alone}$$

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Lifespan Assays

Drosophila melanogaster strains. Animals who undergo the longevity experiments exhibited the same genotype as in genotoxicity assays in order to compare genotoxicity and longevity results. The F_1 progeny from *mwh* and flr^3 parental strains produced by an egg lying of 24 h in yeast was used. Longevity experiments were carried out at 25°C following the procedure of Chavous et al. (2001). Briefly, synchronized trans-heterozygous 72-h larvae were washed and separated into groups of 100 individuals in vials with a mixture of Instant Medium and 4 ml of different concentrations of the 4 substances selected. Emerging adults were collected, anesthetized under CO_2, and placed in 1-ml longevity vials in groups of 10 individuals. Three replicates were used during the complete live extension for each control and concentration established. The survivals were counted and media renewed twice a week.

Statistical analysis of life span. The Kaplan–Meier estimates of the survival function for each control and concentration are plotted as survival curves. The statistical analyses and significance of the curves were assessed by SPSS 15.0 statistics software (SPSS, Inc., Chicago) using the log-rank (Mantel–Cox) method.

Cytotoxicity Assays

Cell culture. The promyelocytic leukemia cell line HL60 was used to assess the cytotoxic effects of juices and phenols. Cells were cultured in RPMI 1640 medium (Biowhittaker, BE12-167F), supplemented with 10% heat-inactivated bovine serum (Biowhittaker, DE14-801F), 200 m*M* L-glutamine (Sigma, G7513), and an antibiotic–antimycotic solution with 10,000 U penicillin, 10 mg streptomycin, and 25 μg amphotericin B per milliliter (Sigma, A5955). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cultures were plated at a density of 25×10^4cells/ml and transferred every 2 d.

Assessment of cell viability. HL60 cells were placed in 12-well culture plates

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

(1×10^5cells/ml) and treated for 72 h with different concentrations of OJ, LJ, hesperidin, and limonene. The cell viability was assessed utilizing the trypan blue exclusion method. Trypan blue (Fluka, 93595) was added to the cell culture with a volume ratio of 1:1. The number of living cells was counted using a hemocytometer under an inverted microscope (Motic, AE30/31) at 100× magnification. Each experiment was repeated in triplicate, growth curves were established, and IC_{50} values were estimated. Curves are plotted as percent survival with respect to control growth at 72 h.

Analysis of DNA fragmentation. In order to detect DNA fragmentation in cells entering apoptosis, HL60 cells (1.5×10^6/ml) were treated with different concentrations of the substances for 5 h. Treated cells were collected, centrifuged at 2500 × g for 5 min. and washed with PBS. DNA was extracted using a commercial DNA extraction kit (Dominion mbl, 243) and was treated with RNase before loading. A final quantity of 1500 ng DNA was subjected to a 2% agarose gel electrophoresis (50 V for 2 h) and stained with ethidium bromide.

RESULTS

Genotoxicity and Antigenotoxicity Testing of Citrus Juices and Components

The SMART assay was used to assess the health-promoting properties of citrus species and its distinctive compounds. Table 1 shows the results for genotoxicity testing of the four substances assayed using SMART. All substances were nonmutagenic at the lowest concentration used. This lower concentration was chosen taking into account the daily food intake for a *Drosophila* larva and giving a similar juice intake to a human consumption of 250 ml/d. Nevertheless, LJ and limonene were mutagenic (0.325 spots/wing) in the SMART at the highest concentration (50% v/v and 0.73 m*M*, respectively). In order to evaluate the recombinogenic potency of mutagenic concentrations, the spots per wing scored in balancer-heterozygous wings were examined where *mwh* clones reflect only somatic point mutations and chromosome aberrations, since somatic recombination is a lethal event. Values of recombinogenicity with respect to the total induced clones were 77 and 62.5% for LJ and limonene, respectively.

Table 2 shows the results for antigenotoxicity assays performed in the combined treatments where larvae were fed chronically with the genotoxicant H_2O_2 (0.15 *M*) and different concentrations of OJ, LJ, or components. Hydrogen peroxide is a well-known mutagen in *D. melanogaster* and has been used to induce microsatellite instability in mismatch repair mutants (López et al. 2002). Hydrogen peroxide induced a mutation rate of 0.45 spots/wing. This result is in agreement with other data using the same genetic background (Anter et al. 2010; Villatoro-Pulido et al. 2009). The antigenotoxic potency of four substances studied against H_2O_2 showed no clear-cut dose-response effect. Average values for the inhibition percent genotoxicity of H_2O_2 were 16.5, 41.6, 47, and 64% for LJ, hesperidin, OJ, and limonene, respectively.

Longevity Assays

Figure 1 shows the survival curves obtained by the Kaplan–Meier method for *Drosophila melanogaster* under chronic treatment with different concentrations of LJ, OJ, hesperidin, and limonene and controls. The entire life-span curves were analyzed statistically by the log-rank (Mantel–Cox) method (data not shown). For controls, average and maximum of entire life span values were 99.2 and 123 d, respectively. Log-rank (Mantel–Cox) analyses for complete life span showed no significant differences between treatment curves and control for OJ. In the case of LJ, curves for higher concentrations (3, 12.5, and 50% v/v) were statistically different from the water control, and the lower concentration (0.75% v/v) ones produced a decrease of life span. Hesperidin curves at 0.15 and 0.06 m*M* were also statistically lower than water control and the lowest concentration (0.0038 m*M*) curves. Finally, 0.0111 and 0.18 m*M* limonene supplementation significantly

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

TABLE 1. Genotoxicity of Lemon and Orange Juices, Hesperidin, and Limonene in the *Drosophila* Wing Spot Test

Compounds	Number of wings	Small spots (1–2 cells) $m = 2$			Large spots (more than 2 cells), $m = 5$			Twin spots, $m = 5$			Total spots, $m = 2$			c
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Negative control (H_2O)	40	6	0.15		0	0		0	0		6	0.15		
Lemon juice (% v/v)														
0.75	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
50.00	40	12	0.3	i(ns)	0	0	–	1	0.02	i(ns)	13	0.325	i(*)	1.354
50.00 *Serrate*	40	3	0.075		0	0		0	0		3	0.075		
Orange juice (%v/v)														
0.75	40	9	0.225	i(ns)	0	0	–	0	0	–	9	0.225	i(ns)	0.937
50.00	40	6	0.15	i(ns)	1	0.02	i(ns)	0	0	–	7	0.175	i(ns)	0.729
Hesperidin (m*M*)														
0.0038	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
0.2400	40	6	0.15	i(ns)	0	0	–	0	0	–	6	0.15	i(ns)	0.625
Limonene (m*M*)														
0.011	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
0.73	40	12	0.3	i(ns)	1	0.02	i(ns)	0	0	–	13	0.325	i(*)	1.354
0.73 *Serrate*	40	5	0.125		0	0		0	0		5	0.125		

Note. No., number of spots; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988); *m*, multiplication factor; *c*, frequency of clone formation per 10^5 cells; +, positive; Serrate, balancer-heterozygous Beaded Serrate genotype wings; –, negative; ns, nonsignificant ($p > .05$), Asterisk indicates significant ($p < 0.05$). The inconclusive and positive data were evaluated by the nonparametric *U*-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995).

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

TABLE 2. Antigenotoxicity of Lemon and Orange Juices, Hesperidin, and Limonene in the *Drosophila* Wing Spot Test

Compounds	Number of wings	Small spots (1–2 cells), $m = 2$			Large spots (more than 2 cells), $m = 5$			Twin spots, $m = 5$			Total spots, $m = 2$			IP (%)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Negative control (H_2O)	40	6	0.15		0	0		0	0		6	0.15		
Positive control (H_2O_2; 0.15 *M*)	40	16	0.4	+(*)	1	0.02	i(ns)	1	0.02	i(ns)	18	0.45	+(*)	
Lemon juice (% v/v)														
0.75	40	13	0.325	i(ns)	0	0	–	1	0.02	i(ns)	14	0.35	i(ns)	22.2
50.00	40	16	0.4	+(*)	0	0	–	0	0	–	16	0.4	+(*)	11.1
Orange juice (% v/v)														
0.75	40	10	0.25	i(ns)	0	0	–	0	0	–	10	0.25	i(ns)	44.4
50.00	40	4	0.1	i(ns)	5	0.125	+(*)	0	0	–	9	0.225	i(ns)	50.0
Hesperidin (m*M*)														
0.0038	40	7	0.175	i(ns)	1	0.02	i(ns)	0	0	–	8	0.2	i(ns)	55.5
0.2400	40	12	0.3	i(ns)	0	0	–	1	0.02	i(ns)	13	0.325	i(ns)	27.8
Limonene (m*M*)														
0.011	40	8	0.2	i(ns)	0	0	–	0	0	–	8	0.2	i(ns)	55.5
0.73	40	3	0.075	i(ns)	1	0.02	i(ns)	1	0.02	i(ns)	5	0.125	i(ns)	72.2

Note. No., number of spots; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988); i, inconclusive data; *m*, multiplication factor; +, positive; –, negative; ns, nonsignificant ($p > .05$), Asterisk indicates significant ($p < 0.05$). The inconclusive and positive data were evaluated by the nonparametric *U*-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995). IP, inhibition percentage.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

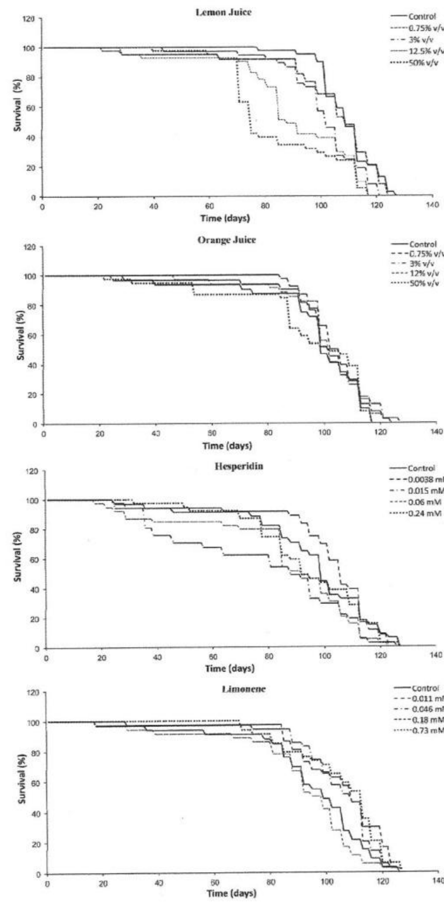


FIGURE 1. Survival curves of *Drosophila melanogaster* fed with different concentrations of lemon and orange juices, hesperidin, and limonene over time.

increased the average life span compared to control flies fed normal food.

In Vitro Human Leukemia Cytotoxicity Assay

A wide range of concentrations was used for every substance (0.625–2.5%, 0.75–20%, 0.37–25 m*M*, and 0.035–2.34 m*M*, for LJ, OJ, hesperidin, and limonene, respectively). Figure 2 shows the relative tumor growth inhibition for the substances assayed. Lemon juice presented an IC_{50} (1.4%) lower than for OJ (4.4%). The concentration-response curves were different for the two juices, with LJ exhibiting a wide plateau for the lower concentrations. Hesperidin and limonene exerted cytotoxic effects on HL60 cells, although the IC_{50} for limonene (0.2 m*M*) was lower than for hesperidin (14 m*M*).

Figure 3 shows the electrophoresis of the genomic integrity in HL60 cells treated for 5 h with different concentrations of the substances. DNA nucleosomal fragmentation was observed in median to highest concentrations of LJ (0.8, 1.2, 1.4, 1.6, 1.8, and 2% v/v) and at the three highest concentrations of limonene (0.6, 1.2, and 2.35 m*M*). This characteristic laddering of apoptotic activity was not observed with OJ or hesperidin.

DISCUSSION AND CONCLUSIONS

The results in the wing spot test for OJ yielded nonsignificant values at the assayed concentrations compared to control. Mutagenicity of OJ was previously found only in the Ames *Salmonella* test using the TA97a and TA98 strains with and without metabolic activation (Franke et al. 2004). In contrast, when the Swiss Webster mouse eukaryotic model was used to carry out the comet assay in peripheral white blood cells, OJ was nongenotoxic (S. I. R. Franke et al. 2005). As *Drosophila* is a eukaryotic model, our results in the wing spot test are in agreement with those of the comet assay in mouse. Lemon juice was tested in the wing spot test of *Drosophila* and data demonstrated a genotoxic inducing recombinogenic activity at the higher concentration (50% v/v). Our findings provide the first result available with respect to the genetic safety of LJ. Hesperidin was nongenotoxic in the SMART assay of

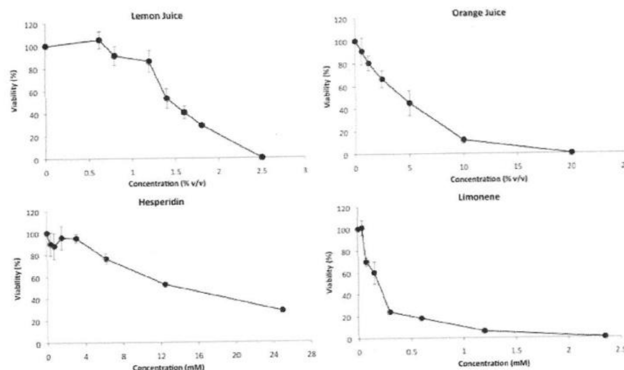


FIGURE 2. Cytotoxicity of lemon and orange juices, hesperidin, and limonene on HL60 cells.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

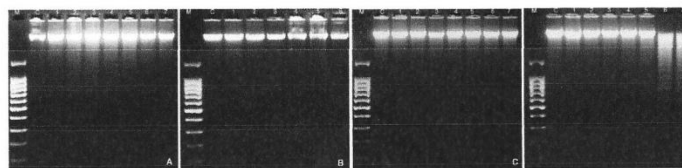


FIGURE 3. DNA fragmentation induced in HL60 cells by lemon and orange juices (A, B), hesperidin (C), and limonene (D). HL60 human leukemia cells were exposed for 5 h to different concentrations of tested compounds. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min. Lemon juice (A): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 0.8 % v/v (lane 2); 1.2 % v/v (lane 3); 1.4 % v/v (lane 4); 1.6% v/v (lane 5); 1.8 % v/v (lane 6); 2 % v/v (lane 7). Orange juice (B): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 1.25 % v/v (lane 2); 2.5 % v/v (lane 3); 5 % v/v (lane 4); 10 % v/v (lane 5); 20 % v/v (lane 6). Hesperidin (C): marker (lane M); control (lane C); 0.39 m*M* (lane 1); 0.78 m*M* (lane 2); 1.52 m*M* (lane 3); 3.12 m*M* (lane 4); 6.25 m*M* (lane 5); 12.5 m*M* (lane 6); 25 m*M* (lane 7). Limonene (D): marker (lane M); control (lane C); 0.037 m*M* (lane 1); 0.075 m*M* (lane 2); 0.15 m*M* (lane 3); 0.3 m*M* (lane 4); 0.6 m*M* (lane 5); 1.2 m*M* (lane 6); 2.35 m*M* (lane 7).

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Drosophila melanogaster, and our results agree with the lack of genotoxicity detected in the *Salmonella* TA98 assay with or without metabolic activation by Van der Merwe et al. (2006). Limonene was not mutagenic in the Ames system using four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) (National Toxicology Program 1990). Nevertheless, our results indicated genotoxic activity for limonene at the higher assayed concentration in the *Drosophila* wing spot test. Our data showed that limonene may produce oxidative stress and ROS generation acting as a pro-oxidant at the highest concentration. This finding agrees with the results in eukaryotic cells that suggest that limonene may act as a pro-oxidant agent dependent upon concentration (Bakkali et al. 2008). Genotoxicity results of the citrus juices and components assayed suggested that limonene content of LJ might be responsible for the recombinagenic activity observed at the highest concentration of LJ. The differential content of limonene in LJ and OJ (86 and 17 mg/L) noted by Maccanore et al. (1998) might reflect an association between genotoxicity induced by LJ and limonene at the higher concentration.

Our antigenotoxicity data for OJ obtained in *Drosophila* against H_2O_2 (47% average percent inhibition) are in agreement with those obtained by S. I. R. Franke et al. (2005), who demonstrated that OJ inhibited DNA damage produced by alkylating agents in the mouse comet assay. Higashimoto et al. (1998) found a 36% mutagenicity-reducing activity of LJ against nitrite-treated 1-methyl-1,2,3,4-tetrahydro-carboline-3-carboxylic acid (MTCCA) using the TA100 strain of *Salmonella typhimurium*. Our results for LJ are also in agreement with the AMES test showing an average percent inhibition of 16.5%. The different antigenotoxic potencies of the OJ and LJ may be related to differential content of antioxidants. It is known that the antioxidant potency of citrus is due to ascorbic acid and phenolic content (Gardner et al. 2000) and that OJ contains higher β-carotene equivalents, ascorbic acid, and total phenolics than LJ (Xu et al. 2008).

The inhibitory capacity of hesperidin against the genotoxic effects of H_2O_2 in the imaginal discs of *Drosophila* was higher at the lowest concentration (55.5%). Kalpana et al. (2009) found hesperidin produced radioprotection by effectively decreasing MN frequency, dicentric aberrations, and comet attributes, and correlated this activity with an ability for ROS scavenging. The higher content of hesperidin in OJ in comparison to LJ, 58 and 20 mg/100 ml, respectively (Cano et al. 2008; Gattuso et al. 2007), may account for the antigenotoxic ability of OJ. Limonene inhibited the genotoxicity of H_2O_2, behaving as a reductor agent that would protect cells from H_2O_2-induced

oxidative stress (Roberto et al. 2009; La Rotta-Hernández, 2007).

The antiproliferative activity of OJ was tested in various K562 (human chronic myelogenous leukemia), HL60 (human leukemia), and MCF-7 (human breast adenocarcinoma) cell lines, showing that a concentration of 10% v/v was able to inhibit 73% HL60 cell growth (Camarda et al. 2007), corresponding to 85% in our experiments. The cytotoxicity of LJ against HL60 cells found in the present investigation was also reported for Caco-2 and HpG_2 cell lines (Lim and Lim 2006; Sun et al. 2002). The cytotoxicity of hesperidin was determined in different cell lines (MDA-MB-435 ER-, MCF-7 ER+, DU-145, HT-29, DMS-114, SK-MEL5) by Manthey and Guthrie (2002), who showed no marked antiproliferative activity due to the glysosylation of the molecule moiety. Nevertheless, many in vivo studies concluded that hesperidin presents anticancer activity in lung, oral, colon, and bladder carcinogenesis (Kamaraj et al. 2009; Tanaka et al. 1997a; 1997b; Yang et al. 1997); these results are in concordance with the in vitro data in the present study performed with HL60 cell line. With respect to the cytotoxicity of limonene, Tatman and Mo (2002) obtained an inhibitory concentration similar to that in the present study (0.18 and 0.20 m*M*, respectively). In vivo assays for limonene are contradictory, as it seems to inhibit the appearance of liver and gastric tumors in mice (Parija and Das 2003; Lu et al. 2004) but Turner et al. (2001) found that limonene induced kidney and bladder tumors in male rats.

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis. The results of our study showed fragmentation of DNA upon treatment of HL60 cells with LJ and limonene, indicating the involvement of apoptosis. A concentration-dependent relationship in the treatment with LJ at lower concentrations was observed. At the two highest doses, it seems that cells are undergoing necrotic processes, which might explain the absence of DNA fragmentation. Limonene induced a slight DNA fragmentation at 0.6 m*M*. This effect was more apparent at highest concentrations (1.2 and 2.35 m*M*) resulting in a concentration-dependent response. These observations may be related to an initiation of the apoptotic process in the tumoral HL60 cells. Limonene seems to act like a pro-apoptotic agent with promising antitumoral properties. Rabi and Bishayee (2009) demonstrated the apoptotic effect of limonene in DU-145 prostate cancer cells but not in normal epithelial prostate PZ-HPV- 7 cells. In human colon cancer cells (SW-480) a DNA fragmentation and induction of caspase-3 by lime volatile oils were noted, which may be due to the involvement of apoptosis mechanism (Patil et al. 2009).

The survival curves for control and the rest of substances at $\geq$75% of living flies were compared. The health span significance was as follows: OJ lower concentration treatments of 0.75 and 3.25% v/v compared to nonenriched diet water control significantly increased health span. Every LJ treatment decreased health span when compared to control, as the low pH of lemon juice (2.3) negatively and differentially affected *Drosophila* adult survival (Mai et al. 2010). A lower concentration of hesperidin (0.0038 m*M*) significantly increased the health span, and the two lowest concentrations of limonene (0.011 and 0.046 m*M*) also significantly improved health span. Taking into account that the maximum average life spans for $\leq$75% survivals are 91, 98, 95, 92, 95, and 92 d for control and the already-mentioned corresponding OJ, LJ, hesperidin, and limonene significant concentrations, a general increase trend was observed in both mean and maximum life span. This implies there was an increase of the health span portion of the life span. Taken together, this study indicated the effects of OJ and LJ on *Drosophila melanogaster* longevity are a result of a combination of antioxidative and prooxidative activities. Given that the fruit fly is an important model for studies on human nutrition and pharmacology, the results of this study suggest that moderate consumption of OJ and its active and predominant components (hesperidin and limonene) may have the potential to strengthen the antioxidant defense system and consequently extend

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

the life span and increase the health span. However, considering the fact that citrus juices may also exhibit pro-oxidant activities toward mitochondria, life-span extension may vary depending upon genetic and environmental factors (Arking 2005).

The results obtained in the present study showed different aspects of the activity of LJ, OJ as well as hesperidin and limonene. Genotoxicity data demonstrated mutagenic activity for LJ and limonene at the highest concentrations. Antigenotoxicity assays indicated that all the genetic safe concentrations are antigenotoxic, showing different percent inhibition. All substances exerted cytotoxic activity, although only LJ and limonene produced DNA fragmentation as an apoptotic mechanism. Finally, as a biological multivariate trait, life-span studies suggested that the lower concentrations of OJ, hesperidine, and limonene increased the health-span part of the life-span curves. Orange juice as a complex mixture and hesperidin and limonene as single compound may be proposed as substances to be studied more extensively as potential nutraceuticals.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

REFERENCES

Abraham, S. K. 1994. Antigenotoxicity of coffee in the *Drosophila* assay for somatic mutation and recombination. *Mutagenesis* 9: 383–86.

Alonso-Moraga, A., and Graf, U. 1989. Genotoxicity testing of antiparasitic nitrofurans in the *Drosophila* wing somatic mutation and recombination test. *Mutagenesis* 4: 105–10.

Anter, J., Campos-Sánchez, J., El Hamss, R., Rojas-Molina, M., Muñoz-Serrano, A., Analla, M., and Alonso-Moraga, A. 2010. Modulation of genotoxicity by extra-virgin olive oil and some of its distinctive components assessed by use of the *Drosophila* wing-spot test. *Mutat Res*. 703: 137–42.

Arking, R. 2005. Gene expression and the extended longevity phenotypes of *Drosophila*. In *Handbook of models for human aging*, ed. M. Conn, 283–98. Elsevier, Burlington, VT.

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. 2008. Biological effects of essential oils—A review. *Food Chem.Toxicol*. 46: 446–75.

Benavente-García, O., Castillo, J., Marin, F. R., Ortuño, A., and Del Río, J. A. 1997. Uses and properties of *Citrus* flavonoids. *J. Agric. Food Chem*. 45: 4505–15.

Camarda, L., Di Stefano, V., Fatta-Del Bosco, S., and Schillaci, D. 2007. Antiproliferative activity of *Citrus* juices and HPLC evaluation of their flavonoid composition. *Fitoterapia* 78: 426–29.

Cano, A., Medina, A., and Bermejo, A. 2008. Bioactive compounds in different citrus varieties. Discrimination among cultivars. *J. Food Composit. Anal*. 21:377–81.

Cerda, S., and Weitzman, S.A. 1997. Influence of oxygen radical injury on DNA methylation. *Mutat. Res*. 386: 141–52.

Chavous, D. A., Jackson, F. R., and O'Connor, C. M. 2001. Extension of the *Drosophila* lifespan by overexpression of a protein repair methyltransferase. *Proc. Natl. Acad. Sci. USA* 98: 14814–18.

Chiba, H., Uehara, M., Wu, J., Wang, X., Masuyama, R., Suzuki, K., Kanazawa, K., and Ishimi, Y. 2003. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *J. Nutr*. 133: 1892–97.

Crowell, P. L. 1999. Prevention and therapy of cancer by dietary monoterpenes. *J. Nutr*. 129: 775S–78S.

Fleming, J.E., Reveillaud, I., and Niedzwiecki, A. 1992. Role of oxidative stress in *Drosophila* aging. *Mutat. Res*. 275: 267–79.

Franke, A. A., Cooney, R. V., Henning, S. M., and Custer, L. J. 2005. Bioavailability and antioxidant effects of orange juice components in humans. *J. Agric. Food Chem*. 53: 5170–78.

Franke, S. I. R., Ckless, K., Silveira, J. D., Rubensam, G., Brendel, M., Erdtmann, B., and Henriques, J. A. P. 2004. Study of antioxidant and mutagenic activity of different orange juices. *Food Chem*. 88: 45–55.

Franke, S. I. R., Prá, D., Erdtmann, B., Henriques, J. A. P., and da Silva, J. 2005.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Influence of orange juice over the genotoxicity induced by alkylating agents: An *in vivo* analysis. *Mutagenesis* 20: 279–83.

Frei, H., and Würgler, F. E. 1988. Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. *Mutat. Res*. 203: 297–308.

Frei, H., Clements, J., Howe, D., and Würgler, F.E. 1992. The genotoxicity of anti-cancer drug mitoxantrone in somatic and germ cells. *Mutat. Res*. 279: 21–33.

Frei, H., and Würgler, F. E. 1995. Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in *Drosophila*. *Mutat. Res*. 334: 247–58.

Gardner, P. T., White, T. A. C., McPhail, D. B., and Duthie, G. G. 2000. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chem*. 68: 471–74.

Garg, A., Garg, S., Zaneveld, L. J. D., and Singla, A. K. 2001. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytother. Res*. 15:655–69.

Gattuso, G., Barreca, D., Gargiulli, C., Leuzzi, U., and Caristi, C. 2007. Flavonoid composition of *Citrus* juices. *Molecules* 12: 1641–73.

González-Molina, E., Domínguez-Perles, R., Moreno, D. A., and García-Viguera, C. 2010. Natural bioactive compounds of *Citrus lemon* for food and health. *J. Pharmaceut. Biomed*. 51: 327–45.

Graf, U., Würgler, F. E., Katz, A. J., Frei, H., Juon, H., Hall, C. B., and Kale, P. G. 1984. Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ. Mutagen*. 6: 153–88.

Graf, U., Alonso-Moraga, A., Castro, R., and Diaz, E. 1994. Genotoxicity testing of different types of beverages in the wing somatic mutation and recombination test. *Food Chem. Toxicol*. 32: 423–30.

Graf, U., Abraham, S. K., Guzmán-Rincón, J., and Würgler, F. E. 1998. Antigenotoxicity studies in *Drosophila melanogaster*. *Mutat. Res*. 402:203–9.

Higashimoto, M., Yamato, H., Kinouchi, T., and Ohnishi, Y. 1998. Inhibitory effects of *citrus* fruits on the mutagenicity of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid treated with nitrile in the presence of ethanol. *Mutat. Res*. 415: 219–26.

Hu, J. J., Dubin, N., Kurland, D., Ma, B. L., and Roush, G. C. 1995. The effects of hydrogen peroxide on DNA repair activities. *Mutat. Res*. 336: 193–201.

Jung, U. J., Lee, M. K., Jeong, K. S., and Choi, M. S. 2004. The hipoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzimes in C57BL/KsJ-db/db mice. *J. Nutr*. 134: 2499–503.

Jung, U. J., Lee, M. K., Park, Y. B., Kang, M. A., and Choi, M. S. 2006. Effect of *citrus* flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. *Int. J. Biochem. Cell Biol*. 38: 1134–45.

Kalpana, K. B., Srinivasan, M., and Menon, V. P. 2009. Evaluation of antioxidant activity of hesperidin and its protective effect on H_2O_2 induced oxidative damage on pBR322 DNA and RBC cellular membrane. *Mol. Cell Biochem*. 323: 21–29.

Kamaraj, S., Ramakrishnan, G., Anandakumar, P., Jagan, S., and Devaki, T. 2009. Antioxidant and anticancer efficacy of hesperidin in benzo(a)pyrene induced lung carcinogenesis in mice. *Invest. New Drugs* 27: 214–22.

La Rotta-Hernández, C. E. 2007. Electrogeneration of hydrogen peroxide applied to the peroxide-mediated oxidation of (*R*)-limonene in organic media. *Electron. J. Biotechnol*. 10: 522–35.

La Vecchia, C., and Bosetti, C. 2006. Diet and cancer risk in Mediterranean countries: Open issues. *Public Health Nutr*. 9:1077–82.

Li, S., Chen, K., Li, X., Zhang, X., and Liu, S. V. 2010. A new cultivation system for studying chemical effects on the lifespan of the fruit fly. *Exp. Gerontol*. 45:158–62.

Li, Y. M., Chan, H. Y. E., Yao, X. Q., Huang, Y., and Chen, Z. Y. 2008. Green tea catechins and broccoli reduce fat-induced mortality in

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Drosophila melanogaster. *J. Nutr. Biochem*. 19: 376–83.

Lim, S. L., and Lim, L. Y. 2006. Effects of citrus fruit juices on cytotoxicity and drug transport pathways of Caco-2 cell monolayers. *Int. J. Pharm*. 307:42–50.

Lindsley, D. L., and Zimm, G. G. 1992. The genome of *Drosophila melanogaster*. San Diego, CA: Academic Press.

López, A., Xamena, N., Marcos, R., and Velázquez, A. 2002. Germ cells microsatellite instability the effect of different mutagens in a mismatch repair mutant of *Drosophila* (spel1). *Mutat. Res*. 514: 87–94.

Lu, G. X., Zhan, L. B., Feng, B. A., Qu, M. Y., Yu, L. H., and Xie, J. H. 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by *d*-limonene. *World J. Gastroenterol*. 10: 2140–44.

Maccarone, E., Campisi, S., Fallico, B., Rapisarda, P. and Sgarlata, R. 1998. Flavor components of Italian orange juices. *J. Agric. Food Chem*. 46: 2293–98.

Mai, W. J., Yan, J. L., Wang, L., Zheng, Y., Xin, Y., and Wang, W. N. 2010. Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in *tilapia (Oreochromis niloticus*) blood cells. *Aquat. Toxicol*. 100: 271–81.

Manthey, J. A., and Guthrie, N. 2002. Antiproliferative activities of *citrus* flavonoids against six human cancer cell lines. *J. Agric. Food Chem*. 50: 5837–43.

Miyagi, Y., Om, A. S., Chee, K. M., and Bennink, M. R. 2000. Inhibition of azoxymethane-induced colon cancer by orange juice. *Nutr. Cancer* 36: 224–29.

Mockett, R. J., and Sohal, R. S. 2006. Temperature-dependent trade-offs between longevity and fertility in the *Drosophila* mutant, Methuselah. *Exp. Gerontol*. 41: 6566–73.

National Toxicology Program. 1990. *Toxicology and carcinogenesis studies of d- limonene (CAS No. 5989-27-5) in F344 rats and B6C3F1 mice (gavage studies)*. National Toxicology Program technical report series no. 347. Bethesda. MD: NIH.

Parija, T., and Das, B. R. 2003. Involvement of YY1 and its correlation with c-myc in NDEA induced hepatocarcinogenesis, its prevention by *d*-limonene. *Mol. Biol. Rep*. 30:41–46.

Patil, J. R., Chidambara Murthy, K. N., Jayaprakasha, G. K., Chetti, M. B., and Patil, B. S. 2009. Bioactive compounds from Mexican lime (*Citrus aurantifolia*) juice induce apoptosis in human pancreatic cells. *J. Agric. Food Chem*. 57: 10933–42.

Rabi, T., and Bishayee, A. 2009.Terpenoids and breast cancer chemoprevention. *Breast Cancer Res. Treat*. 115: 223–239.

Reddy, L., Odhav, B., and Bhoola, K. D. 2003. Natural products for cancer prevention: a global perspective. *Pharmacol. Ther*. 99: 1–13.

Ren, N., Charlton, J., and Adler, P. N. 2007. The flare gene, which encodes the AIP1 protein of *Drosophila*, functions to regulate F-actin disassembly in pupal epidermal cells. *Genetics* 176: 2223–34.

Roberto, D., Micucci, P., Sebastian, T., Graciela, F., and Anesini, C. 2009. Antioxidant activity of limonene on normal murine lymphocytes: Relation to H_2O_2 modulation and cell proliferation. *Basic Clin. Pharmacol*. 106: 38–44.

Selli, S., Cabaroglu, T., and Canbas. A. 2004. Volatile flavour components of orange juice obtained from the cv. Kozan of Turkey. *J. Food Compost. Anal*. 17: 789–96.

Shi, X., Castranova, V., Halliwell, B., and Vallyathan, V. 1998. Reactibve oxygen species and silica-induced carcinogenesis. *J. Toxicol. Environ. Health B* 1: 181–97.

Smith-Warner, S. A., Genkinger, J., and Giovannucci, E. 2006. Fruit and vegetable intake and cancer. In *Nutritional oncology*, eds. D. Heber, G. L. Blackburn, V. L. Go, and J. Milner, 2nd ed. 97–173. Burlington, MA: Elsevier.

So, F. V., Guthrie, N., Chambers, A. F., Moussa, M., and Carroll, K. K. 1996. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer* 262: 167–81.

Downloaded by [Ángeles Alonso-Moraga] at 12:25 27 June 2011

Sun, J., Chu, Y.F., Wu, X., and Liu, R. H. 2002. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* 50: 7449–54.

Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Tanaka, T., and Ogawa, H. 1997a. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* 18: 957–65.

Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara, A., Sumida, T., Fukutani, K., Tanaka, T., Ogawa, H. 1997b. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. *Cancer Res.* 57: 246–52.

Tatman, D., and Mo, H. 2002. Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells. *Cancer Lett.* 175: 129–39.

Tennant, R. W., and Ashby, J. 1991. Classification according to chemical structure, mutagenicity to *Salmonella* and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat. Res.* 257: 209–27.

Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D., and Giammanco, M. 2007 *Citrus* flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chem.* 104: 466–79.

Trotta, V., Calboli, F. C., Ziosi, M., Guerra, D., Pezzoli, M. C., David, J. R., and Cavicchi, S. 2006. Thermal plasticity in *Drosophila melanogaster*: A comparison of geographic populations. *BMC Evol. Biol.* 6: 67.

Turner, S., Tinwell, H., Piegorsch, W., Schmezer, P. and Ashby, J. 2001. The male rat carcinogens limonene and sodium saccharin are not mutagenic to male Big Blue TM rats. *Mutagenesis* 16: 329–32.

Vallejo, F., Larrosa, M., Escudero, E., Zafrilla, M. P., Cerdá, M., Boza, J., García-Conesa, M. T., Espín, J. C., and Tomás-Barberán, F. A. 2010. Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. *J. Agric. Food Chem.* 58: 6516–24.

Van der Merwe, J. D., Jouber, E., Richards, E. S., Manley, M., Snijman, P. W., Marnewick, J. L., and Gelderblom, W. C. A. 2006. A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutat. Res.* 611: 42–53.

Villatoro-Pulido, M., Font, R., De Haro-Bravo, M.I., Romero-Jiménez, M., Anter, J., De Haro-Bailón, A., Alonso-Moraga, A., and Del Río-Celestino, M. 2009. Modulation of genotoxicity and cytotoxicity by radish grown in metal-contaminated soils. *Mutagenesis* 24: 51–57.

Xu, G., Liu, D., Chen, J., Ye, X., Ma, Y., and Shi, J. 2008. Juice components and antioxidant capacity of *citrus* varieties cultivated in China. *Food Chem.* 106:545–51.

Yan, J., Huen, D., Morely, T., Johnson, G., Gubb, D., Roote, J., and Adler, P. N. 2008. The multiple-wing-hairs gene encondes a novel GBD-FH3 domain-containing protein that functions both prior to and after wing hair initiation. *Genetics* 180: 219–28.

Yang, M., Tanaka, T., Hirose, Y., Deguchi, T., Mori, H., and Kawada, Y. 1997. Chemopreventive effects of diosmin and hesperidin on *N*-butyl-*N*-(4-hidroxybutyl) nitrosamine-induced urinary-bladder carcinogenesis in male ICR mice. *Int. J. Cancer* 73: 719–24.

Zordan, M., Graf, U., Singer, D., Beltrame, C., Dalla Valle, L., Osti, M., Costa, R., and Levis, A. G. 1991. The genotoxicity of nitrilotriacetic acid (NTA) in a somatic mutation and recombination test in *Drosophila melanogaster*. *Mutat. Res.* 262: 253–61.