UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS EXPERIMENTALES

DEPARTAMENTO DE CIENCIAS DE LA SALUD

TESIS DOCTORAL

IMPLANTACIÓN DEL HIDROXITIROSOL, TIROSOL Y ESCUALENO EN LA PREVENCIÓN DEL CÁNCER DE MAMA HUMANO. EVALUACIÓN EN MODELOS CELULARES *IN VITRO*

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JAÉN, 29 DE ENERO DE 2016

ISBN 978-84-16819-51-5

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CERTIFICA: Que el trabajo expuesto en la presente Tesis Doctoral titulado"Implicación del hidroxitirosol, tirosol y escualeno en la prevención del cáncer de mama humano. Evaluación en modelos celulares *in vitro"* presentado por D. Fernando Warleta Arias ha sido realizado bajo mi dirección y supervisión, cumpliendo así mismo todas las exigencias para su presentación y defensa para optar al grado de Doctor.

En Jaén, a 11 de Diciembre de 2015

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AGRADECIMIENTOS

ABREVIATURAS

ABTS	Radical catiónico 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ADN	Ácido desoxirribonucleico
AP-1	Activating-protein-1 (proteína de activación 1)
ATP	Adenosín trifosfato
Bax	Bcl-2-associated X protein (proteína X asociada a Bcl-2)
Bcl-2	B cell lymphoma 2 (proteína 2 del linfoma de células B)
Bid	BH3 interacting-domain (dominio de interacción BH3)
B-RAF	Protooncogen de la proteína B-Raf implicada en el crecimiento celular
BRCA2	Breast cancer 2 (Gen supresor de tumores 2)
Ca^{2+}	Ion calcio
Ced-4	Cell death protein 4 (proteína de muerte celular 4)
c-Fos	Protooncogen del factor de transcripción de la familia de los Fos que dimeriza con c-Jun para formar AP-1
c-Jun	Proteína que conforma el factor de transcripción AP-1 junto a c-Fos
C-myc	myelocytomatosis viral oncogene analogue (oncogén análogo al viral de mielocitomatosis)
cyt c	Citocromo c
Cl	Cloro
CO_2	Dióxido de carbono
CO_2	Radical carbonato
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein diacetate

DPPH	Radical 2,2-difenil-1-picrilhidracil
erbB-2	Sinónimo de Her-2/neu
EROs	Especies reactivas de oxigeno
EFSA	European Food Safety Authority (Autoridad Europea de Seguridad Alimentaria)
Fas	Fas cell Surface death receptor (receptor de muerte celular de membrana)
FDA	USA Food and Drug Administration
Her-2/neu	Protooncogen que codifica al receptor 2 de factor de crecimiento epidérmico humano
HDL	High-density lipoprotein (lipoproteína de alta densidad)
HIF	Hypoxia-induced factor (Factor de hipoxia inducido)
H_2O_2	Peróxido de hidrógeno
IC_{20}	Valor de la capacidad inhibitoria a un 20%
IC_{50}	Valor de la capacidad inhibitoria a un 50%
IL-2	Interleuquina 2
K-RAS	Protooncogen de la proteína Ras implicada en la proliferación y transformación de los tumores
LDL	Low-density lipoprotein (lipoproteína de baja densidad)
MCF-7	Línea tumoral de mama humana establecida poco invasiva
MCF10A	Línea epitelial no tumoral de mama humana inmortalizada
MDA-MB-231	Línea tumoral de mama humana establecida altamente invasiva
NFκβ	Nuclear factor kappa beta (factor nuclear kappa beta)
nM	Nanomolar
NAC	N-acetilcisteina
NK	Células Natural killer

NO	Óxido nítrico
O_2	Oxígeno
O_3	Ozono
$O_2^{\cdot -}$	Radical anion superóxido
$^1O_2$	Oxígeno singlete
$^3O_2$	Oxígeno triplete
$^{\cdot}OCl$	Hipoclorito
$^{\cdot}OH$	Radical hidroxilo
$^{\cdot}ONOO$	Peroxinitrito
OMS	Organización Mundial de la Salud
ORAC	Oxygen radical absorbance capacity (Capacidad de absorción de radicales de oxígeno)
p53	Proteína supresora de tumores
PMA	Phorbol Myristate Acetate (Forbol miristato acetato)
ppm	Partes por millón
R^+	Radical alquilo
RAV	Relative Antioxidante Value (Valor antioxidante relativo)
RAW 264.7	Línea establecida de macrófagos murinos
RH	Molécula orgánica
RO^-	Radical alcoxilo
ROO^-	Radical peroxilo
RSA	Radical Scarvenging Activity (Actividad neutralizado de radicales)
SOD	Superóxido dismutasa
SPARC	Secreted Protein Acidic and Rich in Cystein
TE	Trolox equivalente

TNF-α	Tumor necrosis factor –α (factor de necrosis tumoral alpha)
TOC	α-tocoferol
Trolox™	Análogo sintético de la Vitamína E
μM	Micromolar

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INTRODUCCIÓN Y JUSTIFICACIÓN

1. CÁNCER

1.1. Epidemiología del cáncer

El cáncer es actualmente, según el último informe de la Organización Mundial de la Salud (OMS), una de las primeras causas de muerte a nivel mundial. En 2012 se contabilizaron 8,2 millones de muertes por cáncer y un total de 14,1 millones de nuevos casos. Se prevé que los casos anuales aumentarán hasta los 22 millones de nuevos casos en las próximas dos décadas.

Los tipos más frecuentes de cáncer que causan el mayor número de muertes anuales son los de pulmón, hígado, estómago, colon y mama. De estas muertes, se estima que aproximadamente un 30% se deben a 5 factores de riesgo alimenticios y/o de conducta; índice de masa corporal elevado, consumo insuficiente de frutas y verduras, falta de actividad física y consumo de alcohol y tabaco. Por lo tanto son riesgos que pueden prevenirse (IARC, GLOBOCAN 2012)

1.2. Cáncer de mama

El cáncer de mama es el segundo tipo de cáncer más común a nivel mundial, y el primero en incidencia entre las mujeres, con aproximadamente 1,67 millones de nuevos casos diagnosticados en 2012, lo que corresponde al 25% del total de cánceres diagnosticados en ese año. Este tipo de cáncer es el más común en mujeres tanto de regiones desarrolladas como de regiones menos desarrolladas, siendo la incidencia algo más acusada en las regiones menos desarrolladas (883.000 casos) frente a las más desarrolladas (794.000 casos).

Pero en términos de mortalidad, el cáncer de mama es la quinta causa de muerte por cáncer a nivel mundial (522.000 muertes), siendo la causa más frecuente de muerte por cáncer en las mujeres de las regiones menos desarrolladas (324.000 muertes, 14,3% del total) y la segunda causa de muerte por cáncer en las regiones más desarrolladas (198.000 muertes, 15,4% del total), por detrás del cáncer de pulmón.

Se conocen bien varios factores de riesgo del cáncer de mama, aunque en la mayoría de las mujeres afectadas no es posible identificar factores de riesgo específicos (IARC, 2008; Lacey *y cols.*, 2009).

Los antecedentes familiares de cáncer de mama pueden duplicar o incluso triplicar este riesgo. Algunas mutaciones, sobre todo en los genes BRCA1, BRCA2 y p53, se asocian a un riesgo muy elevado de ese tipo de cáncer. Sin embargo, esas mutaciones son raras y explican solo una pequeña parte de la incidencia total de cáncer mamario.

Los factores reproductivos asociados a una exposición prolongada a estrógenos endógenos, así como una menarquia precoz, una menopausia tardía o una edad avanzada de la primera gestación figuran entre los factores de riesgo más importantes del cáncer de mama. Las hormonas exógenas también conllevan un mayor riesgo de cáncer de mama, por lo que las mujeres usuarias de anticonceptivos orales y de tratamientos de sustitución hormonal tienen mayor riesgo de padecer este tipo de cáncer. Como contrapartida, se ha demostrado que la lactancia materna tiene un efecto protector ante este riesgo (IARC, 2008, Lacey *y cols.*, 2009).

La contribución a la carga global de cáncer de mama de diversos factores de riesgo modificables (aquellos que podemos elegir o modificar), suponen el 21% de todas las muertes por cáncer de mama registradas en el mundo. Estas defunciones son atribuibles al consumo de alcohol, el sobrepeso y la obesidad, y la falta de actividad física (Danaei *y cols.,* 2005). Esa proporción fue mayor en los países desarrollados (27%), y el factor más importante fue el sobrepeso y la obesidad. En los países menos desarrollados, la proporción de cánceres de mama atribuibles a esos factores de riesgo fue del 18%, y la falta de actividad física fue el factor determinante más importante (10%).

La diferente incidencia del cáncer de mama en los países desarrollados y los países en desarrollo puede explicarse en parte por los efectos de la alimentación, unidos a la mayor edad del primer embarazo, el menor número de partos y el acortamiento de la lactancia (Peto, 2001). La creciente adopción de modos de vida occidentales en los países menos desarrollados es determinante en el incremento de la incidencia de cáncer de mama en esos países.

1.3. Estrés oxidativo y cáncer

Una importante cantidad de enfermedades crónicas se asocian directa o indirectamente con un estado proinflamatorio inducido y potenciado por el estrés oxidativo (Ad Oleum Habendum, 2011).

El estrés oxidativo se define como el desequilibrio producido en la célula debido a una alta producción de especies reactivas del oxígeno (EROs) y una baja eficiencia del sistema celular para desintoxicar o reparar el daño resultante. Este balance depende directamente de la cantidad de EROs y de antioxidantes dentro de la célula.

El oxígeno molecular no es reactivo, pero sí sus derivados, las EROs. Estos derivados se forman durante el metabolismo aeróbico en el microambiente, y son capaces de interactuar con moléculas de la propia célula, oxidándolas y provocando daños como mutaciones en el ADN, inactivación de proteínas y muerte celular (figura 1).

Se ha demostrado que la presencia de EROs inhibe la capacidad de los quimioterápicos para inducir apoptosis en células tumorales, al mismo tiempo que inhibe la fagocitosis de las células apoptóticas, con el perjuicio que todo ello conlleva. Es importante señalar, que estos compuestos son igualmente producidos por los fagocitos como parte de una respuesta inflamatoria normal contribuyendo a la defensa del organismo.

Las EROs son, a menudo, las causantes de daños producidos en diversas macromoléculas de nuestro organismo como el ADN, los lípidos y las proteínas. Las mitocondrias de las células son las principales productoras de EROs a través de la cadena de transporte electrónico mitocondrial debido a la reducción del oxígeno por un electrón. La probabilidad de que el oxígeno molecular se reduzca a superóxido en vez de agua se ve incrementada si la concentración de protones de la membrana interna es alta y el flujo de electrones de la cadena de respiración es menor.

Pero las EROs también se generan en el retículo endoplasmático o en los fagolisosomas (Freitas *y cols.,* 2002), siendo mayoritariamente en forma de superóxido, aunque la forma más perjudicial es el radical hidroxilo (-OH) derivado del peróxido de hidrógeno (H_2O_2). Las EROs pueden ser generadas en respuesta a diferentes ligandos como hormonas, factores de crecimiento, citoquinas, etc., y son indispensables para mantener la homeostasis celular y en la lucha ante infecciones (Ryter *y cols.,* 1998; Finkel, 1998) así como para actuar de mediadores

fisiológicos, estimulando procesos de transducción de señales para la activación de factores de transcripción. Son necesarias en la expresión génica, crecimiento y proliferación celular, contracción muscular y quimiotaxis (Sen *y cols.*, 1996; Suzuki *y cols.,* 1997; Forman *y cols.*, 1997).

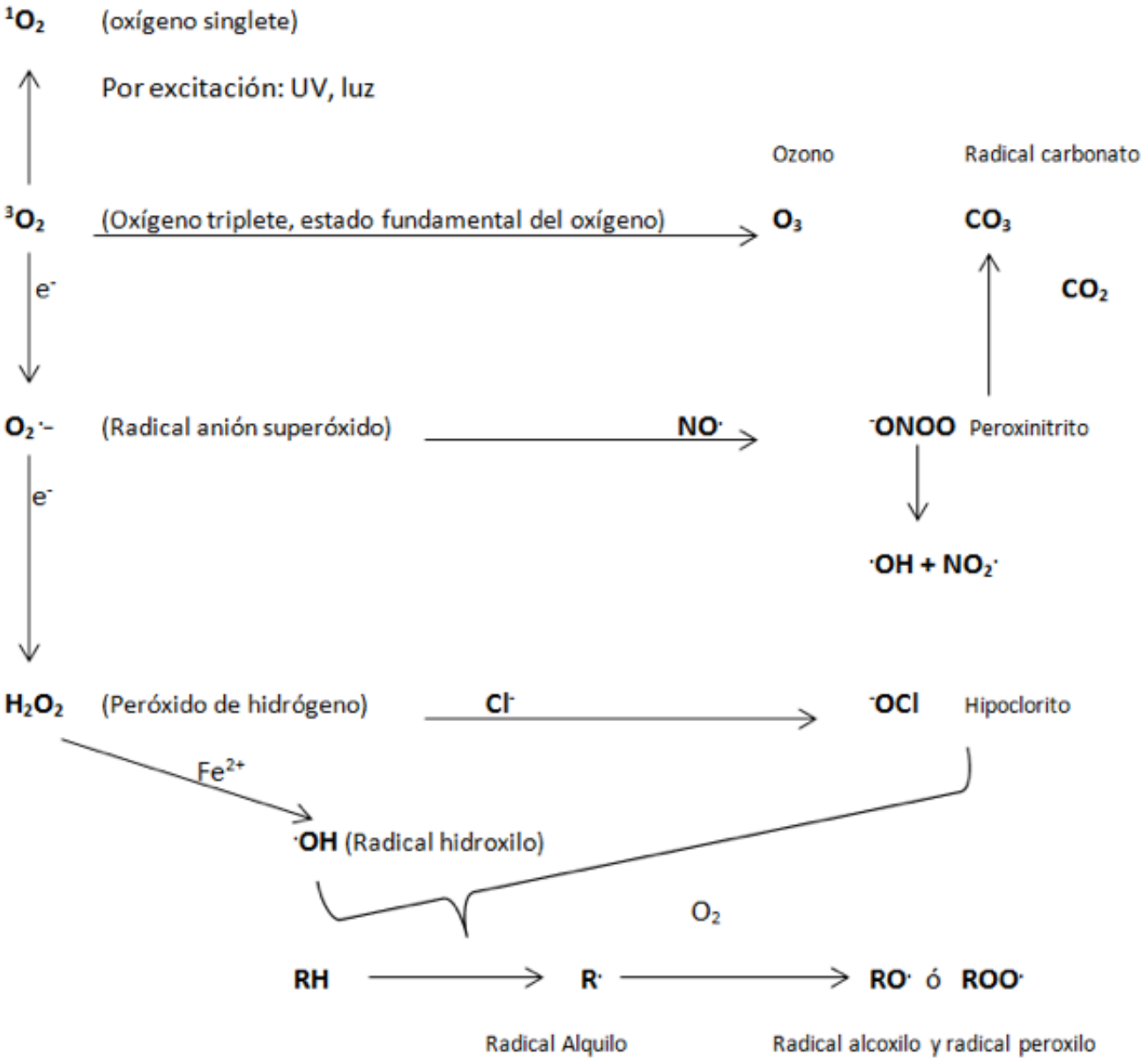


Figura 1. Principales derivados de EROs y componentes orgánicos con los que reaccionan (RH, molécula orgánica) (Bartosz 2009).

El superóxido mitocondrial es principalmente procesado por las superóxido dismutasas a peróxido de hidrógeno (H_2O_2) y oxígeno (O_2). Aunque parte de este H_2O_2 abandona la mitocondria mediante el canal de aniones dependiente de voltaje (saliendo al citoplasma),

parte del H_2O_2 también abandona la membrana por difusión. De manera que podemos encontrar éstos y otros radicales en el citoplasma de la célula, siendo capaces de oxidar otras moléculas orgánicas (Bartosz, 2009). Si se encuentran en concentraciones considerables pueden producir daños en la célula, dando lugar a lesiones importantes. Las células normales se protegen así mismas de estos radicales mediante enzimas endógenos (catalasas, superóxido dismutasas, glutatión peroxidasas) y por antioxidantes (principalmente aportados por la dieta) que se acumulan dentro de ellas mismas.

En la carcinogénesis, las EROs poseen un papel determinante en el daño al ADN necesario para la transformación de una célula normal en tumoral. El daño oxidativo al ADN puede abarcar desde la oxidación específica de purinas y pirimidinas hasta roturas de hebra de ADN, intercambio de cromátidas hermanas o la formación de micronúcleos. Las EROs son capaces de reaccionar con las bases de nucleótidos, modificándolas y dando lugar a alteraciones mutagénicas, que son las principales razones por las que se promueve el cambio de una célula normal a una célula tumoral. Estas lesiones al ADN pueden acumularse dentro de la célula dependiendo del tipo de ERO, su rango de producción y la habilidad celular para proteger o reparar el ADN del daño oxidativo producido.

Las especies reactivas localizadas en el citoplasma también intervienen en la carcinogénesis debido a su capacidad de oxidar las proteínas y lípidos celulares, lo que conlleva la pérdida de la función proteica de las moléculas afectadas. La peroxidación lipídica es una forma de daño oxidativo que tiene lugar en las membranas celulares cuando los ácidos grasos reaccionan con niveles altos de EROs. Dan lugar a radicales de ácidos grasos e hidroperóxidos lipídicos. Estos hidroperóxidos lipídicos causan alteraciones reversibles en la estructura de la membrana y la funcionalidad, además de producir aldehídos reactivos que pueden ocasionar acciones mutagénicas, genotóxicas y citotóxicas. Estas alteraciones pueden prevenirse con los enzimas celulares antioxidantes (endógenos) y con antioxidantes aportados con la dieta (exógenos) que pueden reaccionar rompiendo estos radicales (Zeisel, 2004).

El cáncer es una de las condiciones en las que el cuerpo intenta sacar beneficio de los efectos citotóxicos de las EROs y adaptarse a un nivel alto de éstas, pero superado este nivel, la célula tumoral es mucho más sensible a la muerte celular que una célula normal. Algunos de los agentes quimioterapéuticos más utilizados se basan de hecho en generar radicales libres para promover apoptosis y necrosis en las células tumorales.

Las señales de transducción apoptótica involucran compuestos mitocondriales (como el citocromo c o EROs) bajo el control de factores de regulación (iones intracelulares de Ca^{2+}, balance de proteínas antiapoptóticas Bcl-2 y proteínas proapoptóticas Bax) y una cascada final de ejecución que envuelve a proteasas activadas (caspasas) y endonucleasas de restricción (Lopaczynski y Zeisel, 2001). Uno de los mecanismos de activación de la apoptosis está relacionado con la mitocondria (Schmitt *y cols.,* 2004) *y cols.* 2007). Cuando la mitocondria libera citocromo c, que se une a Ced-4, facilita la escisión de la procaspasa-9, iniciando así el proceso de apoptosis. El proceso se bloquea cuando proteínas de la familia Bcl-2 se liberan al medio. Moléculas antagonistas de Bcl-2, como proteínas tipo Bax o Bid, regulan la apoptosis incrementando la señalización para continuar el proceso apoptótico. Todas ellas actúan dentro de la mitocondria, incrementando los niveles de citocromo c a medida que aumenta la señalización para apoptosis.

Otros mecanismos de activación de la apoptosis involucran factores transcripcionales como el gen c-myc (que activa la apoptosis cuando en el medio no se encuentran determinadas citoquinas) o receptores de muerte celular incluidos en la membrana celular, de la familia de los TNF dónde encontramos receptores de tipo Fas (activan la cascada apoptótica una vez el ligando haya interaccionado con el receptor de membrana).

Generalmente las EROs se consideran productos tóxicos derivados del metabolismo normal de la célula, pero hay evidencias que demuestran que no solo regulan las señales transduccionales de la apoptosis, sino que pueden activar directamente vías apoptóticas (Jabs, 1999). De hecho se ha descrito una regulación transcripcional de las proteínas Fas tras la exposición a oxidantes y se ha visto que pequeños incrementos en la concentración de las EROs o de lípidos peroxidados pueden inducir apoptosis, e incluso antioxidantes como la vitamina E pueden inhibir o retrasar la apoptosis celular.

Hay estudios que relacionan la apoptosis con una peroxidación lipídica de la membrana. La oxidación convierte los aldehídos en moléculas muy reactivas capaces de inducir apoptosis o, incluso necrosis en distintos tipos celulares. Aunque también existen antioxidantes que bloquean la peroxidación lipídica pero no paran la apoptosis (Lopaczynski y Zeisel, 2001). Es lógico pensar que algunos antioxidantes puedan suprimir la apoptosis mientras que otros puede que carezcan de esa habilidad. Probablemente, la acción de estos antioxidantes dependa de la dosis y el tiempo de exposición a los mismos.

Ahora bien, en distintos tipos de líneas celulares humanas tumorales se ha descrito un nivel inferior de enzimas antioxidantes con respecto a sus homólogas de tejido normal (Oberley y Oberley, 1997), lo cual podría otorgar una posible vía de tratamiento creando agentes que aumentaran el estrés oxidativo dentro de la célula para inducir daño al ADN, proteínas y lípidos, así como inducir apoptosis. De hecho Huang *y cols.* (2000) observaron que una inhibición selectiva de la SOD (superóxido dismutasa) inhibia el crecimiento de las células tumorales, pero no el de las normales.

La cantidad de EROs en un organismo puede determinar el riesgo de padecer o no un cáncer. Anteriormente se ha indicado que las EROs son capaces de producir lesiones en el ADN, de hecho producen mutaciones en genes supresores de tumores como puede ser el p53, mutado en más del 50% de los tumores. Por tanto podemos asumir que las EROs pueden actuar como promotores de la carcinogénesis. Además, modulan la actividad de múltiples factores transcripcionales como el factor nuclear kappa beta (NF-κβ) que regula oncoproteínas como Jun y c-Fos, que participan en la iniciación, promoción y progresión del cáncer. Pero se ha descrito que determinados niveles de EROs pueden inhibir la carcinogénesis debido a un incremento de la expresión de p53 y la inducción de la apoptosis en células tumorales (Saeidnia y Abdollahi, 2013).

Un antioxidante es toda aquella molécula capaz de prevenir la oxidación de otras moléculas donando sus electrones a un agente oxidante (como por ejemplo las EROs). De esta manera reducen moléculas oxidantes que pueden producir graves daños a las células y ellas se oxidan, dando lugar a otra molécula menos agresiva que el agente oxidante. Se ha propuesto la acción de estos antioxidantes como quimiopreventivos, pero hay que prestar especial atención a las dosis administradas para tales fines puesto que su efecto podría ser el contrario al deseado dependiendo de la dosis aplicada y de las condiciones en las que se aplique.

Cuando el ADN se daña, la célula se detiene temporalmente en diferentes puntos de control del ciclo celular, donde trata de repararlo, y si la reparación no es posible la célula terminará entrando en apoptosis. La inhibición de esta apoptosis puede derivar en una promoción del cáncer. Las EROs pueden modular la expresión de los genes encargados de la supervisión del ciclo celular y del crecimiento activando o inhibiendo factores de transcripción sensibles al estado de oxidación-reducción celular. De manera que algunos antioxidantes (como la vitamina C) parecen ser capaces de afectar al ciclo celular cuando las células se exponen a un estrés oxidativo, pero una vez que el estrés se pierde, el antioxidante pierde el efecto.

Pero aún con estos resultados no se puede asegurar la acción de los antioxidantes como quimiopreventivos y quimioterápicos, ya que un mismo antioxidante posee diferentes acciones dependiendo de la dosis y el tipo de tumor (Saeidnia y Abdollahi, 2013), por lo cual se necesitan más estudios que aporten claridad al respecto.

Recientemente, se están publicando nuevas investigaciones que ponen en entredicho el efecto beneficioso de los antioxidantes ante el cáncer basándose en los diferentes efectos que tanto las EROs como los antioxidantes tienen sobre organismos y células sanas o tumorales. Así encontramos llamativos resultados que indican como conocidos antioxidantes suplementados en la dieta, como la N-acetilcisteina (NAC) o la vitamina E, aumentan la progresión tumoral y reducen la supervivencia en modelos murinos con cáncer de pulmón inducido por B-RAf- y K-RAS-. Según sus autores, la NAC y la Vitamina E aumentan la proliferación tumoral debido a la disminución de EROs, del daño al ADN y de la expresión de p53 en células tumorales de pulmón de ratones y humanas (Sayin *y cols.,* 2014). Tong L. *y cols.* en 2015 mencionan la extremada complejidad y dificultad que tiene estudiar el papel de las EROs en el cáncer ya que tanto un incremento como una disminución de los niveles de EROs puede ser eficiente en una terapia tumoral teniendo siempre en cuenta la necesidad de mantener unos niveles de EROs intracelulares para la función normal de las células. Lo que sí es evidente es que la producción de EROs en las células tumorales parece ser mayor que en las normales, indicando que han desarrollado algún mecanismo por el cual se hacen tolerantes a esos niveles e incluso obtienen beneficio de ello. Parece ser que un estado prooxidativo persistente en la célula termina en un estrés oxidativo intrínseco con la creación de bastante más H_2O_2 que las células normales. Ciertos factores de regulación celulares pierden su funcionalidad, como la p53, dejando a la célula tumoral en un estado de supervivencia pro-oxidante. Por lo tanto, las células tumorales adquieren un aumento metabólico, ya que necesitan niveles más altos de energía, nucleótidos, lípidos y aminoácidos para mantener constante el crecimiento y la proliferación celular. Por esta razón el metabolismo de una célula tumoral cambia de la fosforilación oxidativa a la glucólisis, ya que con ella se obtiene mayor formación de energía (ATP; adenosín trifosfato), lo que las hace competentes en el crecimiento y desarrollo celular (Saeidnia y Abdollahi, 2013).

En esta última década se ha estudiado el papel de las EROs como mensajeras celulares tanto en la fisiología celular como en las respuestas ante el estrés oxidativo. La célula dispone de mecanismos intrínsecos (enzimas antioxidantes) para regular la concentración de EROs, pero las células tumorales contienen niveles más altos de estas especies reactivas y apenas poseen

enzimas antioxidantes. El no poseer estos enzimas hace plantearse un papel distinto de estas especies oxidantes en la célula tumoral.

La oxidación de la cisteína juega un papel fundamental en la señalización de cascadas redox, de manera que la producción de EROs en respuesta a la oxidación media en la inhibición por contacto célula-célula del crecimiento en fibroblastos y afecta a la unión del ADN con factores nucleares transcripcionales como AP-1 (proteína de activación 1), NF-κβ, p53 e HIF (factor de hipoxia inducido). También se ha descrito la regulación redox de factores de transcripción envueltos en la regulación del ciclo celular, apoptosis y resistencia al estrés oxidativo; así como la interacción de las EROs con la matriz extracelular, la reordenación del esqueleto en la célula y la motilidad, procesos que desencadenan la progresión tumoral y la metástasis (Pani *y cols.,* 2010).

2. ACEITES DE OLIVA

2.1. Historia

La palabra aceite deriva del nombre árabe *az-zait*, que significa "*jugo de oliva"*. Podemos definir el aceite de oliva como el jugo oleoso extraído de la aceituna por prensado en frío, de color verde dorado, denso y de aroma perfumado.

El olivo, es uno de los árboles cultivados más antiguos que se conocen, siendo su cultivo incluso anterior al origen de la escritura (Kiritsakis, 1988). Se remonta a la Era Paleolítica, es decir, hace aproximadamente 12.000 años. Los botánicos creen que el olivo proviene del Asia Meridional pasando después a Egipto, Palestina, Siria y Grecia a mediados de la Edad del Bronce, desde donde fueron propagándolo por toda la cuenca Mediterránea occidental a partir del siglo VI a VII a.C. Los romanos extendieron su cultivo más allá de sus fronteras y lo afianzaron tanto a su cultura que, incluso, consideraban bárbaros a aquellos que usaban grasas animales en vez de aceite de oliva para la alimentación (Camps-Faber, 1984).

Los pueblos mediterráneos que en la antigüedad cultivaban el olivo, no lo empleaban sólo en alimentación, sino que era un elemento higiénico y terapéutico en fricciones tras el baño, purificador en ritos religiosos, útil como combustible para el alumbrado y componente de

pomadas y ungüentos. Como curiosidad, en Babilonia, al médico se le conocía como "asu", que quiere decir "conocedor de los aceites" (Ramis, 2003).

En la Península Ibérica, el desarrollo del cultivo del olivo se remonta a la época de la dominación marítima de los fenicios y ya desde el siglo II se exportaba desde Andalucía (Bética) como lo demuestran los estampados de ánforas encontradas a las afueras de Roma procedentes de Itálica, Astigi y Córdoba.

Figura 2. Colección de la aceituna en un ánfora griega. Figuras negras del S. VI a.C.

Durante la Edad Media la producción. oleícola era continua o intermitente según las épocas, y su uso fue más litúrgico que alimenticio, pero en la España meridional tras la conquista árabe en el siglo VII d.C. se continuó con la actividad aceitera, aunque bajo el control islámico.

Hoy en día, el cultivo del olivo se ha extendido por todos los continentes, excepto la Antártida, encontrándose en lugares tan remotos como China, Vietnam, Oceanía y América y su producción mundial sigue aumentando año tras año.

En la actualidad, España es el país con más cantidad de olivos superando los 300.000.000, seguido de lejos por Grecia e Italia. Asimismo, es el mayor productor y exportador mundial de

aceite de oliva con un continuo incremento de su producción media de las 973.700 toneladas en el 2000/01 hasta los 1.600.000 toneladas en el 2011/12, y es en Andalucía donde se concentra el 83,92% de la producción de ese aceite (Informe de 17 de Octubre de 2012 de economía por países de la Secretaría Ejecutiva del Consejo Oleícola Internacional).

2.2. Alimentación

Una nutrición adecuada se ha considerado la base de una buena salud desde tiempos inmemoriales. En este sentido, Leonardo Da Vinci (1452-1519) afirmó que "la vida de un hombre depende de lo que come".

Y si retomamos a épocas ancestrales, Hipócrates (460-370 a.C.), padre de la medicina moderna, dio las claves de la salud, claves que a día de hoy siguen siendo válidas. Algunas de estas claves relacionadas con la alimentación son:

- Somos lo que comemos
- Que la comida sea tu alimento, y el alimento tu medicina.
- Si encontráramos el modo de que cada persona hiciera la cantidad correcta de ejercicio y recibiera el alimento necesario, ni en exceso ni en defecto, habríamos hallado el camino más seguro hacia la salud.
- La naturaleza no entiende de excesos.

Actualmente se han descrito numerosas patologías relacionadas con la falta de nutrientes en la alimentación, algo que ocurre más a menudo en países subdesarrollados, pero igualmente se ha descrito un gran número de patologías relacionadas con el exceso de nutrientes en la dieta, algo habitual en países desarrollados con un patrón dietético asociado al consumo de grasas, alcohol, carnes, productos elaborados y azúcares.

De este modo, y centrándonos sólo en el cáncer, podemos estimar que cerca de un tercio de los casos de cáncer (entre 3 y 4 millones de casos en el mundo) están causados por una dieta inadecuada (figura 3).

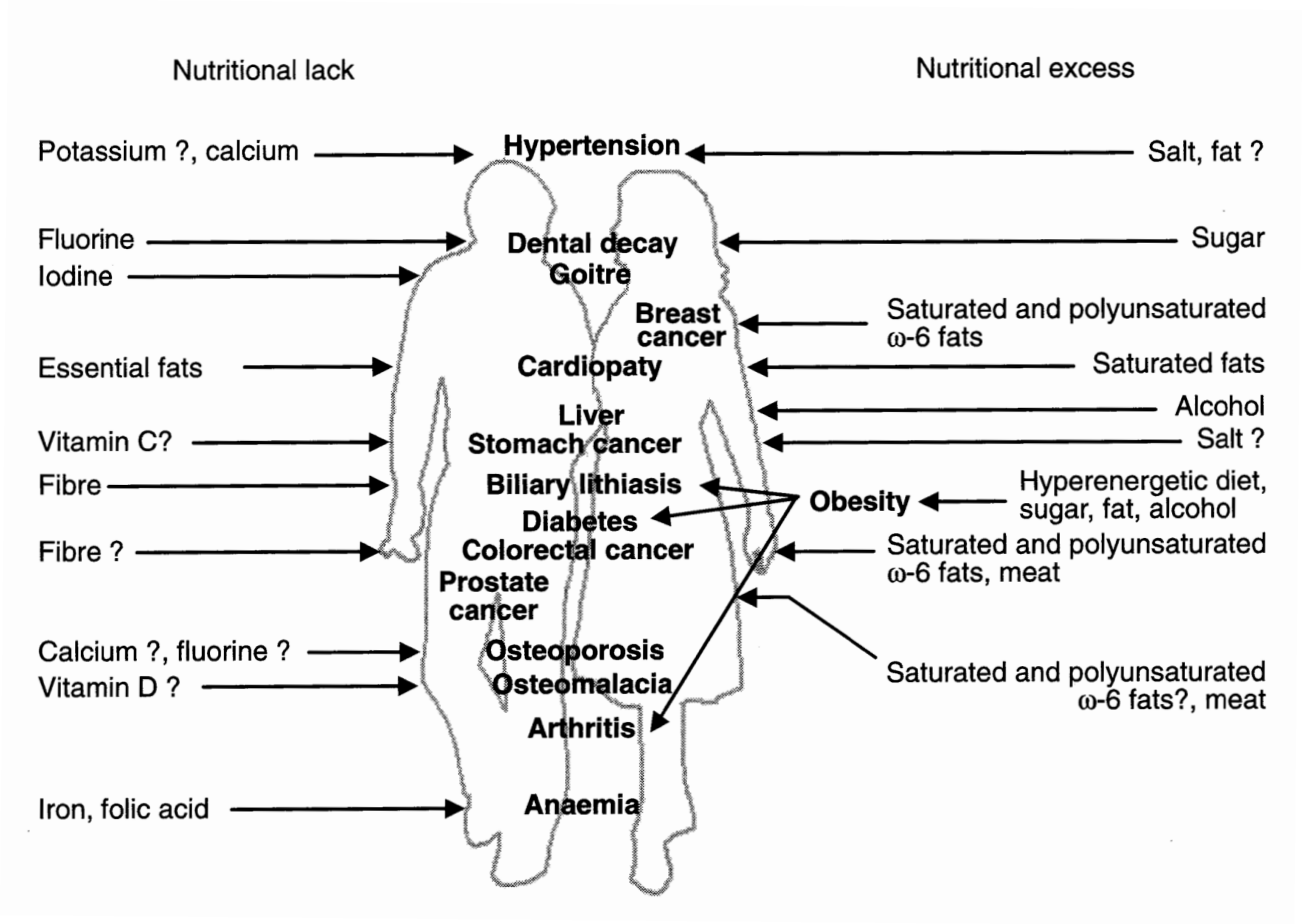


Figura 3. Pathologies associated with nutritional imbalances (James *et al.*, 1990)

2.3. Características de las grasas.

Las grasas son ésteres de glicerina y ácidos grasos. Entre estos ácidos grasos, los más abundantes en los organismos son el palmítico (tripalmitina), esteárico (triestearina) y oleico (trioleina). Mientras que los dos primeros son sólidos a temperatura ambiente, la trioleina se mantiene líquida a temperaturas relativamente bajas, y esta característica es la que diferencia las grasas sólidas de las grasas líquidas o "aceites" (Morros Sarda, 2000).

Las grasas animales empleadas en la industria alimenticia son la manteca de cerdo, el sebo y la mantequilla. Las dos primeras, aunque de composición muy similar a las grasa humana, tienen un coeficiente de digestibilidad bajo. La mantequilla o grasa de la leche tiene una mezcla de varios ácidos grasos estando el oleico en una baja proporción. Al ser sólido a temperatura ambiente hace que aquellos alimentos cocinados con mantequilla sean menos digestibles que

aquellos cocinados con aceite de oliva, además de causar estreñimiento si su consumo es prolongado.

Entre las grasas vegetales se encuentran la manteca de cacao empleada en repostería y los mal llamados aceite de coco y de palma por ser sólidos a temperatura ambiente, que se emplean en la preparación de grasas comestibles y manteca de coco.

Podemos clasificar los aceites en marinos y vegetales. Los marinos se dividen en aceites de pescado, distribuidos uniformemente por los tejidos de la mayoría de los peces; de ballena y foca, extraídos de los tejidos oleaginosos de estos animales y de hígado de bacalao, tiburón, etc., de gran valor terapéutico al ser una importante fuente de vitaminas A y D.

Los aceites vegetales se separan en dos categorías: secantes y no secantes, según se endurezcan o no al ser expuestos a la luz y al aire. Algunos presentan formas intermedias y se denominan semisecantes. Esta propiedad de secado depende de la presencia de ácidos muy insaturados en su composición.

En la categoría de aceites no secantes podemos incluir los aceites de cacahuete, almendras y ricino, con propiedades purgantes, sobre todo el último siendo desaconsejable su uso constante. También se incluye el aceite de colza con componentes sulfurados en su fórmula; los de sésamo, soja y maíz con elevadas concentraciones de ácido linoleico; los aceites de linaza y girasol y por último el de oliva con una composición similar al de girasol pero con diferencias en los ácidos grasos (Morros Sardá, 2000). (Ver figura 4).

2.4. Clasificación

La legislación de la Unión Europea mediante el Convenio Internacional del Aceite de Oliva y de las Aceitunas de Mesa del 2005, define en su Artículo 20 la denominación *"Aceite de Oliva"* al aceite procedente únicamente de la aceituna, con exclusión de los aceites obtenidos mediante disolventes o procedimientos de reesterificación y de toda mezcla con aceites de otra naturaleza.

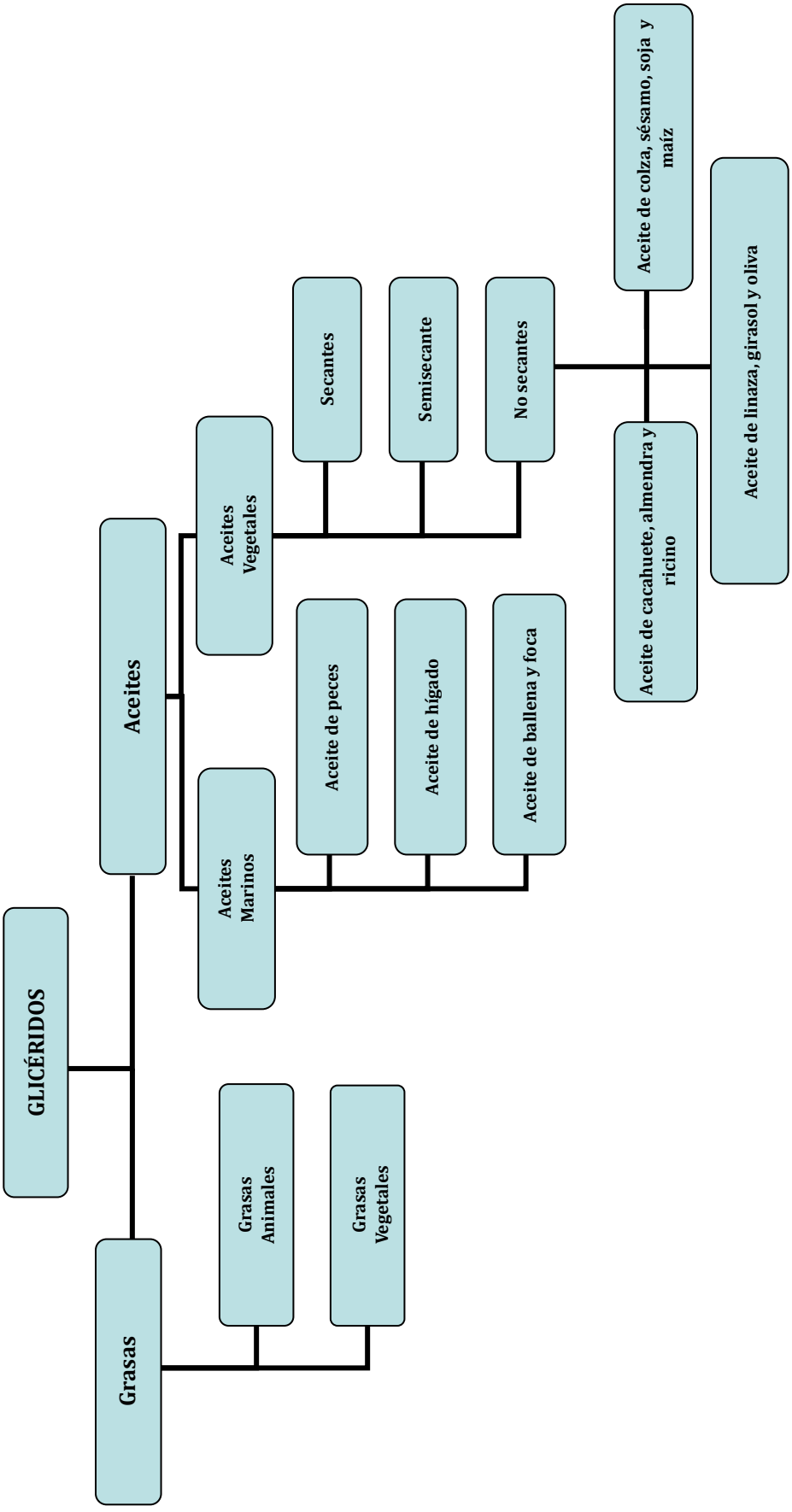


Figura 4. Clasificación de las grasas según su composición en glicéridos.

Esta definición engloba las siguientes denominaciones de aceite de oliva apto para el consumo que quedan recogidas en el Artículo 21 y en su Anexo B:

A) Aceites de oliva vírgenes: aceites obtenidos del fruto del olivo únicamente por procedimientos mecánicos o por otros procedimientos físicos en condiciones, especialmente térmicas, que no produzcan la alteración del aceite y que no hayan tenido más tratamiento que el lavado, la decantación, la centrifugación y el filtrado. Se clasifican y denominan de la siguiente manera:

i) Aceite de oliva virgen extra: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 0,8 gramos por 100 gramos y el resto de sus características corresponden a las previstas para esta categoría.

ii) Aceite de oliva virgen: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 2,0 gramos por 100 gramos y el resto de sus características corresponden a las previstas para esta categoría.

iii) Aceite de oliva virgen corriente: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 3,3 gramos por 100 gramos y el resto de sus características corresponden a las previstas para esta categoría.

B) Aceite de oliva refinado: aceite de oliva obtenido por refino de aceites de oliva vírgenes. Su acidez libre expresada en ácido oleico es como máximo de 0,3 gramos por 100 gramos y el resto de sus características corresponden a las previstas para esta categoría.

C) Aceite de oliva: aceite constituido por una mezcla de aceites de oliva refinados y de aceites de oliva vírgenes aptos para el consumo en la forma en que se obtienen. Su acidez libre expresada en ácido oleico es como máximo de 1 gramo por 100 gramos y el resto de sus características corresponden a las previstas para esta categoría.

(Convenio Internacional del Aceite de Oliva y las Aceitunas de Mesa, 2005. Conferencia de las Naciones Unidas sobre Comercio y Desarrollo. Ginebra, 2005 TD/OLIVE OIL.10/6).

2.5. Composición química y propiedades saludables

El Aceite de Oliva Virgen Extra es el zumo de la aceituna que por su obtención exclusivamente mediante la primera prensa en frío conserva todas sus características físico-

químicas inalteradas y por tanto presenta los mayores valores de aquellos componentes beneficiosos para la salud como las vitaminas A, D, E y K, clorofila, esteroles, tocoferoles y compuestos fenólicos. Otros aceites, como el de girasol, maíz, semillas, etc. cuya composición original podría ser tan rica como la del aceite de oliva virgen, pierden todas sus propiedades en su obtención. En estos casos, el proceso de elaboración comienza con la extracción con disolventes seguido del tratamiento con ácidos para quitar los malos sabores. Tras ello, se elimina la acidez con sosa cáustica a 220-240 °C, lo que elimina todas las vitaminas, se filtra con arcillas activadas para decolorarlo y se destila al vacío para eliminar los malos olores. Por último, se quitan las arcillas (winterización) y se añaden vitaminas sintéticas y colorantes. Todo este proceso hace que el aceite de oliva virgen encabece la lista de los aceites en lo que a calidad y salud se refiere (Frías-Ruiz *y cols.,* 1999).

En la siguiente tabla podemos ver la diferencia en el contenido de ácido oleico de diferentes aceites (Frías-Ruiz *y cols.,* 1999; COI, 2013):

Tabla 1. Contenido en Ácido Oleico de diferentes clases de aceites (COI/T.15/NC nº 3/Rev. 7).

Aceite	**Ácido oleico**
Oliva Virgen y Refinado	55’0 - 83’0
Maíz Refinado	27’5 - 43’0
Girasol Refinado	18’0 - 37’4
Soja Refinado	20’0 - 35’0

La composición del aceite de oliva virgen la podemos dividir en dos grandes bloques:

1. Fracción saponificable: Compuesta por los glicéridos y ácidos grasos, ocupan ente el 97 y el 99% de la composición del aceite. El ácido oleico se encuentra en una proporción de un 56 y un 84% mientras que el linoleico está entre un 3 y un 21%.
2. Los ácidos grasos poliinsaturados, como el linoleico, que es mayoritario en aceites como el de girasol, presentan una baja estabilidad en los metilenos (-CH2-) situados entre dobles enlaces no adyacentes, lo que favorece el inicio del proceso de formación de radicales libres y por tanto, de la oxidación. Los ácidos grasos monoinsaturados, como el caso del ácido oleico, no son tan sensibles al proceso de oxidación, por lo que cuanto

mayor sea el contenido de este último menos oxidable y más estable será el aceite (Frías Ruiz y cols., 1999).

3. Fracción insaponificable: Entre el 1 y el 3% restante en la composición del aceite de oliva virgen, se encuentran más de 230 sustancias (descritas en la actualidad, aunque presumiblemente son más) que le confieren determinadas propiedades, como la protección de la oxidación, entre otras. Estos son:

   - Fosfolípidos: Son el resultado de la unión de un glicerol con dos ácidos grasos y un grupo fosfato. Comprenden entre 40 y 135 mg/kg (Allouche *y cols.,* 2010).
   - Hidrocarburos como el escualeno (entre 800 y 12.000 mg/kg), aproximadamente un 90% del total de hidrocarburos presentes en el aceite de oliva virgen. Similar al beta-caroteno, es un metabolito intermediario en la síntesis del colesterol.
   - Carotenos como luteína y beta-caroteno, precursor de la vitamina A. Actúan como pigmentos del aceite de oliva virgen. Se encuentran entre 1 y 20 ppm.
   - Pigmentos como la clorofila que actúa como antioxidante en oscuridad. Se degrada si se expone a la luz.
   - Tocoferoles (alfa-, beta- y gamma-tocoferol). El alfa-tocoferol o vitamina E es uno de los antioxidantes más importantes del aceite y el más abundante (95 %). Es termolábil, por lo que sólo se conserva si la variedad de aceite se ha obtenido por debajo de los 35 °C. Le confiere estabilidad al aceite.
   - Esteroles como campesterol (1-4%), estigmasterol (0,5-2%), Δ-5-avenasterol (5-20%), ß-sitoesterol (0,7-2,5%) y sitosterol (75-90%). Este último compite con el colesterol en la absorción intestinal. Se presentan libres o esterificados con ácidos grasos.
   - Compuestos fenólicos. Denominados impropiamente a veces “polifenoles” son estructuras orgánicas formadas por anillos aromáticos unidos a derivados funcionales como ésteres, metal ésteres, glicósidos, etc. y unidos a uno o más grupos hidroxilo (OH) que les confieren solubilidad en agua, pudiendo encontrarse, dependiendo de su estructura, en las fases acuosa o lipídica del aceite de oliva virgen. Estos compuestos con una importante capacidad antioxidante son los que le confieren el sabor amargo al aceite de oliva virgen (Gutiérrez-Rosales y cols., 2003).

Este grupo está constituido por un elevado número de compuestos, muchos de ellos aún sin identificar. Se encuentran entre 50 y 500 mg/kg (entre 20 y 50 g/Kg en la pulpa de la aceituna (Artajo LS *y cols.,* 2006)) dependiendo de numerosos factores como la variedad del olivo, el proceso de elaboración, momento de recolección y técnicas empleadas. Los más abundantes y conocidos son los fenoles simples como el hidroxitirosol, tirosol, apigenina, luteolina, ácido vanílico, p-cumárico, ácido felúrico, vanilina, ácido homovainíllico, ácido caféico, así como otros más complejos como oleuropeina, verbacósidos y ligstrósidos (Visioli *y cols.,* 1998; Kris-etherton, 1999). Se ha descrito también la presencia de lignanos como el pinoresinol, acetoxipinoresinol e hidroxipinoresinol en el aceite de oliva virgen (Owen *y cols.,* 2000).

Un reciente estudio ha comprobado el efecto antioxidante de los compuestos fenólicos al observar cómo estos bloquean la oxidación, bien evitando la formación de radicales libres oxidándose ellos mismos, bien actuando sobre los radicales libres ya formados evitando su propagación (Frías Ruiz *y cols.,* 1999) (Ver figura 5).

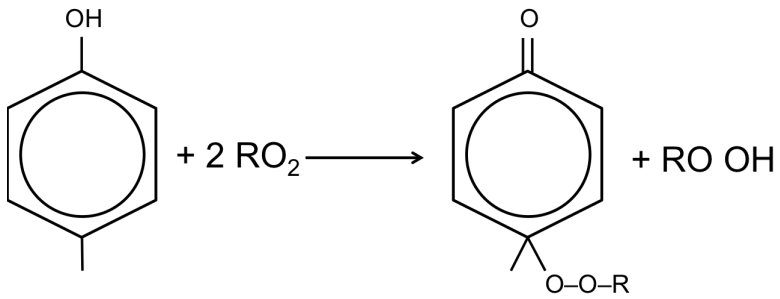


Figura 5. Reacción de los fenoles sobre radicales libres del oxígeno.

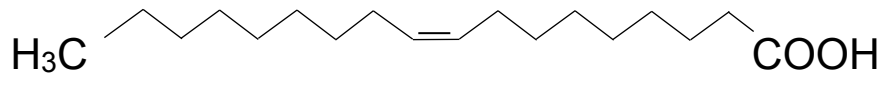
- Alcoholes alifáticos: Gracias a estos alcoholes alifáticos se han podido diferenciar entre aceite de oliva virgen y de orujo (Reglamento de Ejecución nº 1348/2013 de la Unión Europea). A este grupo pertenece el fitol cuya presencia se estima entre 120 y 180 mg/kg. Su presencia es mucho mayor en aceites de orujo de oliva.
- Ceras: Son ésteres de alcoholes alifáticos con ácidos grasos de elevado número de átomos de carbono. Las más frecuentes en el aceite de oliva virgen son las C40, C42, C44 y C46, sin llegar a superar los 350 mg/kg en total.

- Clorofilas y feofitinas: Junto con los carotenoides son los responsables del color del aceite de oliva virgen. En las clorofilas podemos distinguir entre dos tipos ("a" y "b") y sus correspondientes feofitinas ("a" y "b"). El conjunto de esta fracción supone entre 1-20 ppm y su concentración depende del sistema de extracción y de la época de recolección, siendo menor la cantidad a medida que avanza la temporada.
- Componentes volátiles: Son una amplia variedad de sustancias con estructuras muy diversas, cuya característica común es su bajo peso molecular y su baja polaridad. Su carácter volátil les hace responsables de las notas de olor que presentan los aceites de oliva vírgenes. En este grupo se encuentran el 2-metilpropanoato de etilo (olor afrutado), (z)-3-hexenal (verde manzana), (E)-2-hexenal (verde-amargo), entre otros (Allouche *y cols.,* 2010).
- Dialcoholes triterpénicos: Grupo formado principalmente por el uvaol y el eritrodiol cuyas concentraciones en el aceite de oliva virgen son del orden de 8,15 a 85,05 mg/ kg (Allouche *y cols.,* 2009). Aunque se ha visto que la presencia en mayor o menor medida de estos componentes se debe a la variedad de donde se obtenga el aceite de oliva virgen. La determinación del contenido en eritrodiol más uvaol permite detectar fraudes por mezcla con aceites obtenidos mediante extracción con disolventes (Comisión Europea 1991).
- Ácidos triterpénicos: Los compuestos identificados en este grupo se encuentran presentes en el aceite de oliva virgen en un rango que oscila desde 8,90 a 112,36 mg/ kg. Dentro de este grupo se encuentra el oleanólico, maslínico, ursólico, betulínico, 2α-hidroxiursólico y el deoxiursólico. Siendo el ácido oleanólico y el ácido maslínico los más abundantes. Al igual que otros componentes minoritarios el contenido de estos ácidos se ve influenciado por la variedad del cultivo (Allouche *y cols.,* 2009).

2.5.1. Componentes principales.

Dentro de la fracción saponificable, nos centraremos en los tres componentes de mayor relevancia:

- Ácido oleico: Su nombre sistemático es cis-9-Octadecenoico y su estructura es la siguiente:



$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$

Figura 6. Ácido Oléico

Constituye el elemento mayoritario de la fracción saponificable del aceite de oliva virgen y durante mucho tiempo ha sido objeto de estudio de muchos investigadores. Es mucho lo que se sabe de este componente y numerosas las aplicaciones que se le han dado. Vamos a citar algunas de ellas, aunque fundamentalmente nos referiremos a las que están relacionadas con el cáncer de mama.

Como hemos mencionado en numerosas ocasiones, la dieta es un parámetro íntimamente relacionado con numerosas enfermedades. Estudios epidemiológicos demuestran que una alimentación basada en la dieta mediterránea ejerce un papel esencial en la prevención de enfermedades crónicas. Aquí juega un papel importante el aceite de oliva virgen y más en cuanto al ácido oleico se refiere. Se sabe que una dieta rica en este elemento y baja en grasas saturadas (típica dieta mediterránea), tiene efecto protector frente a enfermedades cardiovasculares (de Lorgeril y cols., 2005). Este dato es de gran relevancia, ya que las enfermedades cardiovasculares constituyen la causa de mayor mortalidad en Europa, América y gran parte de Asia (Carrero y cols., 2005). No solamente el ácido oleico actúa como agente preventivo de enfermedades cardiovasculares sino que también ejerce este papel en enfermedades tales como la artritis reumatoide y numerosos tipos de cáncer entre otras (Wahle y cols., 2004).

En cuanto al cáncer de mama se refiere, el ácido oleico se presenta como una opción

en futuras técnicas de tratamiento y prevención de esta enfermedad. Asimismo, estudios recientes demuestran el papel que este componente del aceite de oliva virgen juega en la expresión del gen erbB-2, de forma que el ácido oleico suprime la sobreexpresión de dicho gen constituyéndose un avance más en la lucha contra esta enfermedad (Menéndez y cols., 2005).

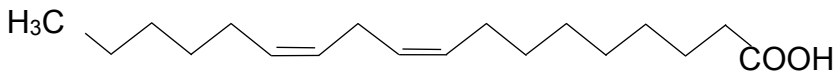
Según determinados trabajos científicos, se sugiere que uno de los mecanismos mediante los cuales se podría iniciar el cáncer de mama sería mediante la epoxidación del 17-β-estradiol. El ácido oleico, entre otros, parece ser que podría actuar como inhibidor de este proceso, de forma que abre otra posibilidad a nuevas medidas terapéuticas (Yu y cols., 2004).

No solamente actúa el ácido oleico en beneficio de nuestro organismo, sino que también tiene efectos sobre determinadas sustancias como, por ejemplo, en los quimioterápicos, como es el Paclitaxel, sobre el cual se sabe que el ácido oleico tiene efectos potenciadores en su citotoxicidad sobre células tumorales de cáncer de mama (Menéndez y cols., 2001). Algo similar podría ocurrir con otro quimioterápico muy utilizado que es la Doxorubicina (Germain y cols., 1998).

Otro aspecto a destacar del ácido oleico es el efecto que ejerce sobre el crecimiento o desarrollo de células tumorales de cáncer de mama. Experimentos realizados in vitro revelan que a determinadas concentraciones, el ácido oleico puede estimular o inhibir el crecimiento de células tumorales, de modo que a concentraciones de 0.25 μg/ml, el ácido oleico estimula el desarrollo celular, mientras que a concentraciones mayores los efectos son inversos (Rose y cols., 1990). Asimismo, los efectos del ácido oleico en la adhesión de células tumorales se hacen también evidentes según determinados investigadores, de tal forma que el ácido oleico se presenta como un potenciador de la adhesión celular en líneas metastásicas de tumores de mama, con las consecuencias que conlleva esto en el proceso metastásico (Johanning y cols., 1995).

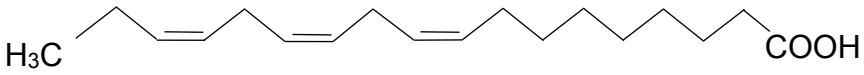
Son numerosas las investigaciones realizadas sobre este elemento esencial en la dieta mediterránea y presente abundantemente en el aceite de oliva virgen. De todas ellas, hay un aspecto muy claro y evidente, y es el efecto protector que tiene frente a una enfermedad tan relevante en la sociedad como es el cáncer de mama (Franceschi y cols., 1996).

- Ácido linoleico y linolénico: Sus nombres sistemáticos son cis,cis-9,12-Octadecadienoico y todo-cis-9,12,15-Octadecatrienoico respectivamente y sus estructuras son las siguientes:



$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$

Figura 7. Ácido linoleico



$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$

Figura 8. Ácido linolénico

Ambos se encuentran formando parte del aceite de oliva virgen, aunque en menor proporción que el ácido oleico. Sin embargo, dotan al aceite de oliva virgen de algunas propiedades relevantes al igual que el oleico. Por citar alguna de estas propiedades, el ácido linoleico contribuye a las propiedades antioxidantes que tiene el aceite de oliva virgen (Kelawala y cols., 2004), además de tener también efectos en el crecimiento de células tumorales de cáncer de mama inhibiendo el desarrollo celular o induciéndolo en función de la concentración, y de promover fenómenos necróticos y apoptóticos en las células tumorales (Maggiora y cols., 2004).

Al igual que el ácido oleico, el linoleico parece tener efectos en los fenómenos de adhesión celular, de forma que ésta se ve incrementada por este componente, (Johanning y cols., 1995) por lo que su papel en el proceso metastásico sería también fundamental.

Hay que hacer también referencia a las propiedades que ejerce frente a enfermedades cardiovasculares, ya que actúa como preventivo respecto a ellas constituyéndose como otro de los elementos esenciales en la dieta mediterránea (de Lorgeril y cols., 2005).

En cuanto al ácido linolénico se refiere, también puede tener relevancia en tumores de mama, de modo que puede ejercer un papel regulador en los fenómenos de adhesión

celular (Watkins y cols., 2005) a través de la regulación en la secreción de SPARC (Secreted Protein Acidic and Rich in Cystein). Está demostrado su papel en la modulación de la estructura y función de receptores esteroideos (Kenny y cols., 2001) y parece ser que también tiene efectos potenciadores de la citotoxicidad en algunos quimioterápicos como el Tamoxifeno (Kenny y cols., 2000).

Estas y otras muchas propiedades que confieren estos tres elementos hacen del aceite de oliva virgen un alimento con bastante atractivo dentro del mundo científico y culinario, un alimento digno del lugar que ocupa en la dieta mediterránea, y digno de despertar mayor interés científico día a día.

Estudios epidemiológicos y experimentales sugieren que algunos nutrientes, incluidas las sustancias fenólicas, juegan un papel importante en la prevención de varias enfermedades. Sin embargo, la extensa distribución de sustancias fenólicas en los aceites vegetales naturales se ve drásticamente reducida tras el procesamiento para hacerlos comestibles (Gracian, 1986).

Trabajos realizados anteriormente con ratones en laboratorio, muestran cómo una alimentación rica en aceite de oliva virgen suprime la actividad celular de las natural killer (NK) (Yaqoob y cols., 1994), la proliferación de mitógenos estimulados (Yaqoob y cols., 1995) y la expresión de receptores para IL-2 y transferrina (Jeffery y cols., 1995) en preparaciones linfocitarias esplénicas. La comparación del efecto de alimentar con aceite de oliva virgen, de girasol y de girasol enriquecido en ácido oleico sobre el sistema inmune ha sugerido que los efectos son debidos tanto a la presencia de ácido oleico como a componentes no lipídicos contenidos en el aceite de oliva virgen (Yaqoob y cols., 1998).

2.5.2. Componentes minoritarios.

Los beneficios fisiológicos conferidos al aceite de oliva virgen se ven probablemente determinados por una gran cantidad de componentes minoritarios en la fracción insaponificable, entre los que se incluyen carotenos, fenoles, esteroles y triterpenos (Duthie *y cols.,* 1989).

Algunos de los antioxidantes llamados "polifenoles" en el aceite de oliva virgen pueden tener la habilidad de destruir sustancias que lideran la proliferación de células cancerígenas. Evidentemente, la investigación es necesaria para resolver de qué modo se produce esta protección, pero la evidencia muestra que las mujeres en los países mediterráneos padecen menos cáncer de mama que en países como Estados Unidos y Australia, donde el porcentaje es muy alto.

Entre los efectos específicos de los compuestos fenólicos del aceite de oliva virgen que promueven la salud, se incluyen la inhibición de la oxidación de lipoproteínas de baja densidad (LDL) que se cree que están implicadas en el inicio de la arterosclerosis (Fitó *y cols.,* 2000; Visioli *y cols.,* 2002), la reducción de los niveles de radicales libres (Visioli *y cols.,* 1998), la reducción de la inflamación por inhibición de la actividad lipooxigenasa, reduciendo la generación de prostaglandinas y disminuyendo la generación de EROs por los leucocitos (de la Puerta *y cols.,* 1999), y la potenciación de la respuesta mediada por macrófagos (Visioli *y cols.,* 1998).

El principal componente fenólico presente en el aceite de oliva virgen es la oleuropeína, y sus efectos han sido estudiados en numerosas investigaciones, como el efecto en la composición lipídica del corazón de rata (Muriana *y cols.,* 1992; Manna *y cols.,* 2004), el efecto citotóxico en células tumorales humanas de la cavidad oral (Babich *y cols.,* 2003), el aumento de la respuesta de los macrófagos murinos mediante un aumento de la producción de óxido nítrico (Visioli *y cols.,* 1998) o la prevención de la inflamación en ratas ovariectomizadas (Puel *y cols.,* 2004), etc.

La actividad biológica de los esteroles y triterpenoides ha sido también estudiada en una variedad de proyectos como el desarrollo y control del tracto reproductor en el hombre (Gómez *y cols.,* 1997), la actividad antioxidante, antiarterosclerótica y antihipertensiva de los triterpenoides del aceite de oliva virgen en ratas (Somova *y cols.,* 2003), el efecto vasodilatador de dos triterpenoides sobre la aorta de ratas (Rodríguez-Rodriguez *y cols.,* 2004), la acción inhibidora del beta-sitoesterol de la producción de radicales libres por los macrófagos RAW 264.7 (Moreno, 2003), etc.

Cada vez es mayor el número de estudios que se centran en otros componentes minoritarios de aceite de oliva virgen como son el escualeno, el tirosol, el hidroxitirosol, etc. cuya actividad beneficiosa para el organismo y preventiva ante una variedad de enfermedades ya ha sido planteada y estudiada con resultados muy positivos. Son estas investigaciones las que sugieren

que componentes minoritarios como el hidroxitirosol son capaces de inhibir la proliferación de células tumorales de colon al estimular la liberación de citocromo c (cyt c) desde las mitocondrias y con ello inducir su apoptosis (Della Ragione *y cols.,* 2000), o comparan la capacidad citotóxica y citoestática de algunos de estos componentes minoritarios sobre células (Sáenz *y cols.,* 1998).

Entre las acciones más importantes que se le puede atribuir a la fracción minoritaria del aceite de oliva virgen se encuentra la actividad antitumoral, como ha demostrado Owen *y cols.* (2000) en estudios anteriores con escualeno, quercetina e hidroxitirosol o como demostraron Ragione y cols. (2000) al describir la inducción de la apoptosis dependiente del citocromo c por el hidroxitirosol.

Desde hace muchos años, el escaso prestigio que poseía el aceite de oliva virgen como alimento saludable ha llevado a que fuese utilizado en investigaciones en animales y humanos como placebo, suministrando dosis de aceite de oliva virgen y dosis de otros aceites como, sobre todo, el de pescado, comparando sus efectos para demostrar las diferentes propiedades beneficiosas en el organismo. Pero la mayoría de esos trabajos concluyen con que el aceite estudiado no tiene el efecto propuesto o afirman que no hay evidencias que demuestren efectos diferentes del aceite en cuestión comparado con el placebo. La explicación no se halla en que el potencial saludable del aceite de pescado y otros sea inferior al que creemos, sino más bien a que el placebo tiene, por lo menos, las mismas propiedades estudiadas que el aceite de pescado, no encontrándose diferencias significativas entre el tratamiento con uno u otro aceite. Una carta editada en *The Lancet* sugiere que "estudios futuros sobre suplementación con aceite no deberían considerar al aceite de oliva virgen como placebo" (Milner, 1989).

Afortunadamente, cada vez se va abandonando más la errónea idea de que el aceite de oliva virgen puede emplearse como placebo a la hora de estudiar los efectos de otros aceites en diferentes campos de la ciencia. Artículos recientes como "Randomised double-blind placebo-controlled trial of fish oil in the treatment of depresion" demuestran esta afirmación al concluir el estudio del efecto del aceite de pescado sobre pacientes con depresión con que *"no hay evidencias de que el aceite de pescado comparado con el placebo* [aceite de oliva virgen] *mejore el ánimo a pesar del incremento circulatorio de ácidos grasos poliinsaturados omega-3"*. Asimismo, en su conclusión, afirma también que *"el estado de ánimo mejoró en ambos grupos de pacientes dentro de las dos primeras semanas de tratamiento"*, lo que apoya la idea de que no es un buen placebo (Silvers *y cols.,* 2005).

2.5.3. Fenoles simples: Hidroxitirosol y Tirosol

El hidroxitirosol es el componente fenólico del aceite de oliva virgen más antioxidante. La variedad autóctona de Jaén (variedad Picual) tiene una concentración medio / alta de hidroxitirosol (300 ppm) (Brenes *y cols.,* 1999). Se ha descrito su capacidad para inducir apoptosis en células tumorales de colon y en leucemias mieloides (Ragione *y cols.,* 2000).

El tirosol, uno de los componentes más abundantes, junto con el hidroxitirosol, de la fracción insaponificable del aceite de oliva virgen, previene la oxidación de LDL protegiendo a los microtúbulos de la oxidación de los filamentos de actina (Giovannini *y cols.,* 1999).

Actualmente se está realizando un estudio en el Houston Methdost Cancer Center liderado por la Dra. Tejal Patel en el que investigan el potencial efecto del hidroxitirosol sobre mujeres pre- y postmenopáusicas para prevenir el cáncer de mama, basando su investigación en la relación establecida entre la densidad de tejido mamario (se considera denso cuando en su mayoría está formado por tejido fibroso o glandular y hay menos proporción de tejido adiposo) y el riesgo a padecer cáncer de mama. La Dra. Patel trata de investigar en 100 pacientes con una toma diaria de hidroxitirosol durante 12 meses, si este compuesto del aceite de oliva virgen es capaz de reducir la densidad de los tejidos mamarios y con ello disminuir el riesgo de padecer cáncer de mama. (Tejal Patel MD).

2.5.4. Hidrocarburos: Escualeno.

El Escualeno es un hidrocarburo. Dentro de la fracción insaponificable del aceite de oliva virgen es el componente más abundante (6600 ppm). Su principal uso terapéutico es la posibilidad de su actuación como terapia adyuvante en una variedad de cánceres (Gregory *y cols.,* 1999). Está demostrado que el escualeno contenido en el aceite de oliva virgen, y su consumo, reduce el riesgo de diversos tipos de cáncer (Newmark *y cols.,* 1997; Rao y cols., 1998), así como que inhibe el crecimiento tumoral (Gregory *y cols.,* 1999). Otras propiedades son la disminución de la peroxidación lipídica en la superficie de la piel humana y el papel que juega en la salud de la retina (Fliesler *y cols.,* 1997).

Nakagawa *y cols.* (1985) estudiaron el efecto combinado del escualeno con agentes quimioterápicos en sistemas tumorales murinos observando un fuerte efecto potenciador de la actividad antitumoral.

2.5.5. Propiedades saludables de los aceites de oliva.

Estas son algunas de las conclusiones recogidas y ratificadas en el Documento de Consenso del primer Congreso Internacional Sobre Aceite de Oliva y Salud (CIAS 2004), celebrado en Jaén, donde más de 300 científicos de todo el mundo trataron las investigaciones y evidencias científicas sobre los efectos saludables y protectores del aceite de oliva virgen:

- Los estudios epidemiológicos muestran que una dieta mediterránea, rica en aceite de oliva virgen, disminuye el riesgo cardiovascular.
- La dieta mediterránea, rica en aceite de oliva virgen, mejora los principales factores de riesgo cardiovascular, incluyendo el perfil lipídico, la presión arterial, el metabolismo de la glucosa y el perfil antitrombótico. Además, dicha dieta modula positivamente la función endotelial, la inflamación y el estrés oxidativo. Algunos de estos efectos se atribuyen a los componentes menores presentes en el aceite de oliva virgen, por lo que en la definición de dieta mediterránea debería incluirse dicho alimento.
- En países con poblaciones que mantienen la típica dieta mediterránea, y donde el aceite de oliva virgen es la principal fuente de grasa, la incidencia de cáncer es menor que en los países del Norte de Europa.
- Los estudios más recientes apoyan de modo consistente que la Dieta Mediterránea, basada en el consumo de aceite de oliva virgen, se acompaña de un envejecimiento saludable y aumenta la longevidad. Sin embargo, a pesar de los significativos avances producidos en los últimos años, se requieren más investigaciones para conocer los mecanismos específicos implicados en dichos efectos, así como la contribución de los distintos componentes del aceite de oliva virgen en sus beneficios.

En 2004, la FDA (U.S. Food and Drug Administration) calificó el aceite de oliva virgen como alimento saludable al reducir el riesgo de enfermedades coronarias. Sustentados en datos científicos contrastados como los perfilados por el "Interim Procedures for Qualified Health Claims in the Labeling of Conventional Human Food and Human Dietary Supplements" del FDA,

homologan la certeza de esta afirmación tanto para el aceite de oliva virgen en sí, como para aquellos alimentos que lo contengan.

La FDA sugiere tomar aproximadamente dos cucharadas de aceite de oliva virgen diariamente ya que puede reducir el riesgo de padecer enfermedades coronarias debido a la grasa monoinsaturada que posee. Para que este efecto beneficioso sea posible, el aceite de oliva virgen debe sustituir a una cantidad similar de grasa saturada y no aumentar el número total de calorías ingerido diariamente.

Nuestro organismo requiere una dieta equilibrada y saludable en la cual estén presentes todos los nutrientes en su justa medida. Las grasas son entre estos nutrientes, unos de los más importantes ya que son una fuente primordial de energía por el elevado contenido calórico que poseen (ver tabla 2) e intervienen en el desarrollo de diversas estructuras celulares y en la asimilación de vitaminas y minerales entre otras funciones vitales.

Tabla 2: Aporte calórico generado por un gramo de diferentes nutrientes

Un gramo de materia	**Valores calóricos fisiológicos aproximados**
Grasas	9
Proteínas	4
Hidratos de Carbono	4

Las grasas son esenciales, sin embargo no todas son beneficiosas para el organismo, ya que algunas ejercen un efecto perjudicial, como son las grasas saturadas (Owen y cols., 2004) o las altamente perjudiciales grasas vegetales trans, grasas sometidas al proceso de hidrogenación o adición de hidrógeno a altas presiones y temperaturas en presencia de un catalizador, transformando el ácido graso cis en trans y obteniendo como consecuencia un producto sólido a temperatura ambiente con mayor resistencia térmica.

De este grupo de grasas no beneficiosas se excluyen las grasas poliinsaturadas y monoinsaturadas, como el ácido oleico, contenido en el aceite de oliva virgen, grasas que van a ser aprovechadas por el organismo y que reducen los niveles de colesterol en sangre entre otros efectos saludables que conlleva su ingesta (Michalsen y cols., 2005).

Existen otras muchas circunstancias por las que se deben incluir en la dieta grasas de origen vegetal como el aceite de oliva virgen. Las grasas, una vez en el intestino, por diferentes mecanismos inhiben la motilidad gástrica y la liberación del jugo gástrico. Esta acción, a veces, es muy acusada y hasta puede resultar perjudicial si el consumo de grasas, como las animales, ha sido excesivo. En cambio, el aceite de oliva virgen ejerce esta acción de una forma más suave y además produce un retraso en la evacuación gástrica de forma que aseguramos un ataque hacia los alimentos por parte del jugo gástrico.

Estudios epidemiológicos muestran que las poblaciones que consumen una dieta mediterránea predominantemente a base de vegetales, poseen menores incidencias de enfermedades crónicas que aquellos que consumen una dieta típica del Norte de Europa o Norteamericana. Este efecto ha sido atribuido al mayor consumo de frutas y verduras y al menor consumo de productos animales, y en particular su grasa. Aunque la ingesta total lipídica en poblaciones mediterráneas es más alta que en otras regiones, la mayor proporción procede del aceite de oliva virgen y no de grasas animales. El aumento del consumo de aceite de oliva virgen implica una reducción de enfermedades cardiovasculares, artritis reumatoide, y, en menor grado, una variedad de cánceres.

Otro motivo fundamental por el cual una dieta equilibrada exige el aporte de grasas, entre ellas y esencialmente el aceite de oliva virgen, es por su contenido en vitaminas liposolubles. El aceite de oliva virgen, generalmente, es pobre en vitamina A, sin embargo, es muy rico en vitamina E. Aunque este excepcional alimento sea pobre en vitamina A hemos de resaltar que es un magnífico solvente para la misma, por lo que la adición de dicha vitamina al aceite proporcionaría sin duda un producto mucho más aceptable que otros aceites como el de pescado. De todas formas, aunque el aceite de oliva virgen contenga poca vitamina A y carezca de vitamina D, por su acción colagoga o estimulante de la secreción biliar, es un vehículo excepcional para la absorción de la totalidad de vitaminas liposolubles que se ingieren con el resto de los alimentos. Además, el aceite de oliva virgen gracias a la secreción biliar es emulsionado, y por los jugos pancreáticos y entéricos es saponificado y desdoblado, por lo que su absorción es inminente, con un coeficiente de digestibilidad de 97.8 (Morros Sardá, 2000).

En la actualidad hay una gran preocupación por el incremento de la obesidad en la población de los países desarrollados (Baschetti, 2005). Según un estudio realizado en Gerona, una alimentación basada en la dieta mediterránea está íntimamente relacionada con el Índice de Masa Corporal y con la obesidad, lo que conllevaría a una reducción de la misma (Schroder y

cols., 2005). Por esto, es obvio que el aceite de oliva virgen estará entre las soluciones que se propongan frente a este problema de salud.

El ácido oleico, presente en una proporción de un 80 % en el aceite de oliva virgen virgen, aumenta la relación entre HDL / LDL que son las siglas de High Density Lipoprotein o "colesterol bueno" y Low Density Lipoprotein o "colesterol malo". Se diferencian en la lipoproteína que los transporta, lo que se traduce en un efecto protector del organismo ya que las HDL transportan el colesterol hasta el hígado donde será eliminado. Esto evita que la LDL acumule el colesterol en tejidos como en las paredes internas de las arterias formando placas de ateroma, que son las responsables de la arteriosclerosis y en última instancia de la trombosis arterial e infartos. El ácido oleico actúa tanto aumentando el nivel de HDL como disminuyendo el de LDL, a diferencia de otros aceites como el de semilla que actúa indistintamente en la reducción de HDL y LDL.

Otros de los problemas de salud que pueden derivar de una dieta desequilibrada, son los cardiovasculares, tales como la arteriosclerosis e hipertensión. El aceite de oliva virgen, como hemos referido antes, es rico en vitamina E, mediante la cual determinados mecanismos reducen la producción de prostaglandina E_2 de forma que ejerce un efecto protector frente a los problemas arterioscleróticos (Dayong *y cols.,* 2004). Por otro lado y según un seguimiento realizado por la universidad de Navarra, una dieta rica en aceite de oliva virgen proporciona una barrera protectora frente a un exceso de presión sanguínea (Alonso *y cols.,* 2004). Además, el aceite de oliva virgen es un elemento antioxidante en sí, de forma que está demostrado su papel en la disminución del estrés oxidativo (Moreno *y cols.,* 2001; Tur Mari, 2004). Las enfermedades arteriosclerósicas aparecen en gran medida en individuos con una elevada cantidad de colesterina en sangre y los niveles de esta grasa en sangre están muy condicionados por la cantidad de colesterina de los alimentos ingeridos. La mayoría de las grasas animales que se usan en alimentación contienen gran cantidad de colesterina. Sin embargo, el aceite de oliva virgen no contiene colesterina, aunque sí contiene un análogo llamado fitosterina, pero que se elimina por las heces no afectando a la colesterinemia y ayudando a otras de las virtudes reconocidas del aceite que es la regulación intestinal (Morros Sardá, 2000).

El aceite de oliva virgen es un estimulante del crecimiento óseo y de la absorción del calcio y su mineralización, contribuye a la regulación de la glucosa en la sangre, disminuye la tensión arterial, regulariza el funcionamiento del aparato circulatorio, ejerce un efecto protector y

tónico sobre la piel y mejora el metabolismo al actuar sobre el sistema endocrino. A estas propiedades hay que sumar que el elevado contenido en antioxidantes, como la vitamina E, lo convierte en un ingrediente altamente recomendado tanto en la infancia como en la vejez.

Según la teoría de la peroxidación de los radicales, el envejecimiento y la muerte celular serían tan solo la consecuencia de la oxidación mediada por los radicales libres. El envejecimiento empezaría desde el momento del nacimiento y constituiría el resultado de los daños que se ocasionan en las células a lo largo de toda la vida. Teniendo en cuenta que las grasas poliinsaturadas son la diana fundamental de los radicales libres, es necesario efectuar una elección prudente de nuestras grasas alimenticias con el fin de "conservar" al máximo los fosfolípidos (contienen ácidos grasos) de las membranas celulares. Como la grasa más rica en ácidos grasos monoinsaturados (menos susceptibles al ataque de los radicales libres) es el aceite de oliva virgen, entendemos que es la elección más conveniente, ya que cada vez son más los trabajos que demuestran experimentalmente que su consumo ayuda a alcanzar una larga esperanza de vida.

En un estudio realizado por Trichopoulos (1995) se observó cómo, en un grupo de 2.368 mujeres, el consumo de aceite de oliva virgen se asociaba directamente a una reducción del riesgo de cáncer de mama. En opinión del oncólogo Ramón Colomer, el 80% de los casos de cáncer no son debidos a causas genéticas sino a malos hábitos de la salud, como la dieta, cuyo papel preventivo en la evolución de la enfermedad puede ser tanto o más importante que los mismos fármacos antitumorales (Colomer, 2004). En células de cáncer de mama, se ha comprobado que en aquellas tratadas con ácido oleico se reduce aproximadamente un 50% la expresión del oncogén Her-2/neu clave en la etiología y progresión del cáncer (Menéndez *y cols.,* 2005). Para el doctor Colomer, esta propiedad del aceite de oliva virgen ofrece un novedoso mecanismo molecular por el cual se podría regular el comportamiento maligno de las células de cáncer de mama y por lo tanto ser de ayuda en el diseño de futuros estudios epidemiológicos (Menéndez *y cols.,* 2005).

Experimentos realizados en humanos muestran como gracias a la ingesta de aceite de pescado junto con aceite de oliva virgen, pacientes con artritis reumatoide pudieron mejorar su movilidad mejor que con aceite de pescado solo o con otros aceites (Berbert *y cols.,* 2005).

Se ha demostrado que la ingesta de aceite de oliva virgen también modula la función inmune, y en particular los procesos inflamatorios asociados con el sistema inmunológico. El aceite de oliva virgen es un componente dietético antioxidante, y la atenuación del proceso

inflamatorio que produce podría explicar sus efectos beneficiosos sobre el riesgo de ciertas enfermedades, ya que la oxidación y los procesos inflamatorios parecen ser la base de factores en la causa de estas enfermedades en el hombre. Los efectos antioxidantes del aceite de oliva virgen son probablemente debidos a una combinación entre su alto contenido de ácido oleico y su contenido de una variedad de antioxidantes vegetales, en particular oleuropeina, hidroxitirosol, y tirosol. (Wahle *y cols.,* 2004). Estos componentes, entre otros, modulan positivamente el proceso de oxidación que deteriora las células y la función del endotelio, así como la inflamación, implicada en infinidad de problemas de la salud, lo que conlleva a un menor riesgo de padecer arteriosclerosis, Parkinson, Alzheimer, demencia vascular, deterioro cognitivo, diabetes y cáncer, a lo largo de la vida.

El aceite de oliva virgen también juega un importante papel en la diabetes. Se ha demostrado que aquellas personas que incluyen aceite de oliva virgen en su dieta, tienen un mejor control sobre su diabetes y niveles más bajos de ciertas grasas en la sangre al compararla con la dieta rica en carbohidratos que normalmente se recomienda para este tipo de diabetes (Sharafetdinov *y cols.,* 2003; Zamora Ardoy *y cols.,* 2004).

Pero la ingesta de aceite de oliva virgen por sí sola, no es la solución para las diferentes enfermedades, sino que su consumo favorece el buen funcionamiento del organismo y previene de las patologías causadas por una descompensada nutrición calórica como es el exceso de grasas animales. El equipo del Dr. Faine ha demostrado recientemente cómo la combinación de la ingesta de aceite de oliva virgen junto a una restricción calórica en la dieta ejercía una acción defensiva mucho más acentuada en la prevención de enfermedades cardiacas que tan solo la suplementación con aceite de oliva virgen en la dieta (Faine *y cols.*, 2004).

2.6. Aceite de oliva y cáncer

El estilo de vida que seguimos está estrechamente relacionado con el desarrollo de multitud de enfermedades, y entre ellas los cánceres. Se estima que el 80% de los cánceres en humanos, especialmente el de mama, ovario, próstata, colorrectal, tracto digestivo y pulmón, se deben a estas circunstancias.

Son numerosos los estudios epidemiológicos que llegan a la conclusión mencionada anteriormente de que las poblaciones que siguen una dieta Mediterránea, como en España;

Italia o Grecia, en la que la principal fuente de grasas es el aceite de oliva virgen, tienen una menor incidencia de cáncer que aquellos países que no la siguen como en el norte de Europa, América del norte o Australia. Las evidencias científicas publicadas hasta la actualidad nos revelan que el consumo de aceite de oliva virgen tiene una influencia favorable sobre la iniciación, promoción y progresión de la carcinogénesis, asociándose también con un menor grado de malignidad clínico e histopatológico. El efecto preventivo que se le atribuye podría estar mediado por diferentes mecanismos como los cambios en la composición y estructura de las membranas celulares, los cambios en la biosíntesis de eicosanoides o en las vías de señalización intracelular, la modulación de la expresión génica, la reducción del estrés oxidativo celular y del daño sobre el ADN o la modulación del sistema inmunitario y del balance hormonal, clave en determinados tipos de cáncer hormono-dependientes como los de mama o próstata (Escrich E *y cols.,* 2006)

2.6.1. Hidroxitirosol, Tirosol y Escualeno *vs* Cáncer

Dado que las EROs están inmersas en la etiología del desarrollo de muchos tumores, cada vez son más numerosas las investigaciones que tratan de elucidar la relación entre la capacidad antioxidante de determinados componentes minoritarios del aceite de oliva virgen y su acción frente a diversos tipos de cánceres.

En este sentido, autores como Owen *y cols.* en el 2000 fueron de los primeros en sugerir que los fenoles presentes en el aceite de oliva virgen, como el hidroxitirosol y tirosol, son potentes inhibidores de la formación de radicales libres previniendo el cáncer colorrectal. Moreno *y cols.* en el 2003 acotaron que el tirosol disminuye la producción de EROs en macrófagos de manera dosis-dependiente tras una estimulación con PMA y Smith T.J. en el año 2000 recopiló la información conocida hasta el momento del potencial efecto quimiopreventivo del escualeno en su review “Squalene: potential chemopreventive agent”, aunque con posterioridad algunos autores han postulado una falta de capacidad quimiopreventiva de estos compuestos ante la tumorigénesis como Scolastici C *y cols.* en 2004 acerca del escualeno y su nula capacidad para inhibir la hepatocarcinogénesis en ratas Wistar.

3. CULTIVOS CELULARES EN LA INVESTIGACIÓN BÁSICA DEL CÁNCER

Aunque las cuestiones sobre el crecimiento celular y sobre la inducción del cáncer son, en última instancia, preguntas sobre el comportamiento de células individuales dentro de un organismo vivo, por razones prácticas y éticas, muchos de los potenciales fármacos antitumorales son inicialmente testados en células que crecen en cultivo. De este modo, se puede controlar y modificar el medio en el que crecen estas células, se puede definir claramente el tipo de célula diana que queremos estudiar, pueden ser estudiados y analizados los cambios acontecidos en estas células después del tratamiento testado, tanto con agentes carcinógenos como anticancerígenos, y por último, se puede determinar el destino de estas sustancias dentro de la célula, sus mecanismos bioquímicos de acción y sus vías de entrada en la célula diana. También pueden ser manipuladas genéticamente para observar las consecuencias a diferentes niveles de la alteración de uno o varios genes problema [Lodish *y cols.,* 1995]. Por estas razones, los estudios del crecimiento celular normal, así como del tumoral y de la inducción del cáncer dependen en gran medida de la utilización de células en cultivo como base para los estudios posteriores en modelos animales y en humanos.

4. JUSTIFICACIÓN.

• Existe un creciente interés por consumir alimentos “naturales” y “saludables”, y el aceite de oliva virgen lo es.

• Diferentes evidencias epidemiológicas demuestran que el consumo habitual y moderado de aceite de oliva virgen podría ayudar a prevenir el desarrollo de determinadas enfermedades crónicas y/o degenerativas como el cáncer (fundamentalmente, de mama, colon y próstata).

• En la fracción insaponificable del aceite de oliva virgen se encuentran los componentes minoritarios, que son constituyentes extranutricionales, presentes en bajas concentraciones pero, según los últimos datos científicos, es en ellos donde podría residir, en gran medida, el efecto preventivo anteriormente reseñado.

• No obstante, aún no conocemos en profundidad los mecanismos moleculares que expliquen estos efectos, aunque algunos de ellos se han asociado con efectos antioxidantes.

• La cantidad e incluso calidad de los componentes minoritarios es variable en los diferentes aceites de oliva virgen dependiendo de varios factores, fundamentalmente: de la variedad, grado de maduración de la aceituna y, método de elaboración y obtención del aceite (Allouche *y cols.,* 2007, 2009).

• La Unión Europea a través de la EFSA (European Food Safety Authority), regula las alegaciones nutricionales y de salud en el etiquetado de alimentos.

• Para ser autorizadas estas alegaciones, tienen que tener una base científica contrastada.

• En noviembre de 2004, la FDA autorizó la alegación de salud que hace referencia a que el consumo diario de una cantidad moderada de aceite de oliva virgen podría reducir el riesgo de enfermedad cardiaca coronaria debido a la presencia de ácidos grasos monoinsaturados en el aceite de oliva virgen.

• Recientemente, el gobierno Español ha solicitado a la EFSA la aprobación para Europa de alegaciones de salud similares a las aprobada por la FDA.

• Para que en el futuro, se puedan solicitar nuevas alegaciones nutricionales y de salud que puedan aparecer en el etiquetado del aceite de oliva virgen, es necesario que profundicemos en el estudio de los efectos sobre la salud de los componentes minoritarios. Esto representará el sustento científico para solicitar dichas alegaciones.

• La EFSA incluye los estudios realizados *in vitro* sobre células humanas como un primer paso para aportar datos sobre los mecanismos moleculares que los componentes nutricionales pueden activar en nuestro organismo con el fin de emitir hipótesis científicas que después tienen que ser corroboradas en ensayos clínicos.

• Todo ello repercutirá de forma directa y beneficiosa en el sector oleícola ante la posibilidad de poder aparecer en el etiquetado de los aceites de oliva, alegaciones nutricionales y de salud.

• La diferenciación de sus aceites de oliva es una prioridad para algunas empresas que están dispuestas a invertir en estos estudios científicos para diferenciar sus aceites de oliva desde una perspectiva biosaludable.

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

Los compuestos minoritarios presentes en los aceites de oliva vírgenes podrían estar implicados en la prevención del cáncer de mama en humanos.

Por su importancia cuantitativa, los compuestos fenólicos hidroxitirosol y tirosol, junto con el hidrocarburo, escualeno, podrían tener un papel central en este proceso preventivo.

OBJETIVOS

El principal objetivo de este trabajo es el de elucidar la participación o responsabilidad del hidroxitirosol, tirosol y escualeno en los efectos preventivos atribuidos al aceite de oliva virgen frente al cáncer de mama.

Para ello, los objetivos parciales marcados en este trabajo han sido:

Objetivo 1: Evaluar las propiedades antitumorales *in vitro* del hidroxitirosol, tirosol y escualeno en los modelos tumorales humanos de mama MCF-7 y MDA-MB-231.

Objetivo 2: Estudiar la capacidad preventiva *in vitro* del desarrollo de tumores del hidroxitirosol, tirosol y escualeno en el modelo celular humano de mama MCF-10A.

Objetivo 3: Definir las propiedades antioxidantes del hidroxitirosol, tirosol y escualeno, tanto *per se,* como su acción antioxidante intracelular comparada entre modelos celulares tumorales humanos de mama MCF-7 y MDA-MB-231 y no tumoral humano de mama MCF-10A

Objetivo 4: Determinar la capacidad del hidroxitirosol, tirosol y escualeno de prevenir el daño oxidativo al ADN.

TRABAJOS EXPERIMENTALES

Los formatos originales de las publicaciones que comprenden esta tesis se encuentran en los anexos. A continuación se expone el contenido literal de estos trabajos en un formato más amigable para su lectura.

Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells.

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Keywords: Squalene; human breast cancer; comet assay; virgin olive oil; cancer prevention.

Abbreviations: AAPH, 2,2'-azobis (2-methylpropionamidine) dihydrochloride; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); CPT, camptothecin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; EMCCD, electron-multiplying charge coupled device; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; FL, fluorescein; HBSS, Hank's balanced salt solution; HuMEC, human mammary epithelial cells medium; LMA, low melting point agarose; MEM, minimum essential medium; NEAA, non-essential amino acids; NMA, normal melting point agarose; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffer saline; PI, propidium iodide; PMS, phenazine methosulphate; ROS, reactive oxygen species; RPMI, Roswell park memorial institute 1640 medium; SQ, squalene; TOC, tocopherol; TR, trolox; XTT, (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate).

Abstract

Until now, very little is known about the effect of Squalene (SQ) on human breast tumourigenesis and its antioxidant capacity. In the present work we investigated the SQ scavenging properties and its effect on cell proliferation, cell cycle profile, apoptosis, reactive oxygen species (ROS) level and oxidative DNA damage using human breast cell lines. Our results showed that SQ neither posses scavenging activity nor alter significantly cell proliferation rates, cell cycle profile or cell apoptosis on human mammary epithelial cells (MCF10A), minimally invasive (MDA-MB-231) and highly invasive (MCF7) breast cancer cells. However, we found that SQ exerted the following effects on MCF10A epithelial cells in a dose-dependent manner: (a) it decreased intracellular ROS level; (b) it prevented H_2O_2-induced oxidative injury and, finally, (c) it protected against oxidative DNA damage. Interestingly, SQ did not exert these effects on MCF7 and MDA-MB-231 cancer cells. Therefore, our data suggest that SQ, found in high amounts in virgin olive oils, could be partially responsible for the lower incidence of breast cancer in populations that consume the Mediterranean diet by their protective activity against oxidative DNA damage on normal mammary cells.

Introduction

Olive oil is the principal source of fat in the Mediterranean diet (Pérez-Jiménez et al., 2005). Consumption of olive oil has been associated with low incidence and prevalence of cancer, including breast cancer (Escrich et al., 2006; Owen et al., 2000a). In this regard, it has been suggested that the lower risk of breast cancer, associated with high olive oil consumption, may be due to the presence of squalene (SQ) (Newmark, 1997).

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane) is a triterpenic hydrocarbon present at high concentrations in shark liver or virgin olive oils. In virgin olive oil, its content range from 0.8 to 13 g/kg (Allouche et al., 2007; Cert et al., 2000; Owen et al., 2000b) being the major olive oil hydrocarbon (more than 90%). SQ, an intermediate metabolite in cholesterol metabolism, is considered as a remarkable bioactive substance since several interesting biological activities have been reported. Among them, antioxidant and antitumour properties have been described (Owen et al., 2000a). Indeed, *in vitro* experimental evidences

indicate that this compound is a highly effective singlet oxygen scavenging agent (Saint-Leger et al., 1986), and a chemopreventive agent against chemically-induced cancer (Smith et al., 2000; Sotiroudis and Kyrtopoulos, 2008). Moreover, SQ is able to inhibit aberrant hyperproliferation, an event that precedes mammary tumourigenesis *in vivo* (Katdare et al., 1997), and to play a tumour-inhibiting role in animal models (Murakoshi et al., 1992; Newmark, 1997; Rao et al., 1998).

Despite evidences suggesting its anticarcinogenic and antitumour properties, there are no available studies about the effects of SQ either on human breast tumour or human mammary epithelial cells. Therefore, the aim of this paper was to investigate the possible antioxidant effect of SQ and its relation with the antiproliferative capacity and oxidative DNA damage protection on both, human breast cancer cell lines (MCF7 and MDA-MB-231), and immortalized non-tumourigenic human mammalian epithelial cell line (MCF10A). For this purpose, we studied SQ scavenging activity, cell proliferation, cell cycle profile, apoptosis, intracellular oxidative stress and DNA oxidative damage.

2. Materials and methods

2.1. Materials

Hepes Buffer, Sodium Pyruvate, Non-Essential Amino Acids mixture 100x (NEAA), DCFH-DA, Dimethyl sulfoxide (DMSO), 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium salt) purity ≥ 90%, 5-Methylphenazinium methyl sulfate, N-Methylphenazonium methyl sulfate (PMS) purity ~ 98%, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane (Squalene CAS 111-02-4 (SQ)) purity ≥ 98%, DL-all-rac-α-Tocopherol (Vitamin E CAS 10191-41-0 (TOC)) purity ≥ 96%, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity ≥ 97%, 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) purity ~ 97%, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) purity ~ 90%, (S)-(+)-camptothecin (CAS 7689-03-4 (CPT)) purity ~ 95%, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt tablets (CAS 30931-67-0 (ABTS)), PBS and HBSS were purchased from Sigma-Aldrich Co. (St Louis, MO). Minimum essential medium with Eagle's salts (MEM), Foetal Bovine Serum (FBS) and Phenol-

Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express, HuMEC Ready Medium kit, Fluorescein (FL) and Propidium Iodide (PI) were obtained from Invitrogen (Eugene, OR). $K_2S_2O_8$ (CAS 7727-21-1) was from Panreac Quimica S.A.U. (Barcelona, Spain). Culture plates were from NUNC^{tm} (Roskilde, Denmark). PI/RNase Staining Buffer kit, FITC-conjugated Annexin V and Binding Buffer were from BD Biosciences Pharmigen (San Diego, CA.). Comet assay kit was from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD.).

2.2. Estimation of radical scavenging activity by the DPPH test

The antioxidant activity of SQ was measured against the stable radical DPPH, as previously reported (Brand-Williams et al., 1995) with some modifications. Briefly, 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of SQ in 96-well plates at 0.06, 0.13, 0.25, 0.5 and 1 moles of antioxidant/moles of DPPH. α-tocopherol (TOC) was used as a standard antioxidant control and a sample without antioxidant was also measured as a blank control. The decrease of the absorbance at 520 nm was determined immediately and every 5 min until 2 h in a microplate reader (TECAN, GENios Plus). Measurements were performed in triplicate.

The inhibition of the DPPH radical was calculated according to the percentage of Radical Scavenging Activity (% RSA) formula:

$$\% \text{ RSA} = [(A_{C\,(0)} - A_{A\,(t)}) / A_{C\,(0)}] \ 100$$

where $A_{C\,(0)}$ is the absorbance of the control at t = 0 min and $A_{A\,(t)}$ is the absorbance of the antioxidant at t = 50 min.

2.3. ABTS radical scavenging assay

The ABTS cation radical scavenging activity was determined using a previously reported procedure (Re et al., 1999). ABTS radical ($ABTS^{+\cdot}$) was obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{+\cdot}$ was diluted in ultrapure water

until absorbance at 734 nm was 0.7 (± 0.1). SQ and TR (as antioxidant reference) was dissolved in ethanol to 10 mM stock solution and diluted with ultrapure water to the assayed concentrations. 20 µl of each concentration of SQ or standard (TR), blank (ultrapure water) or ethanol control (8 %) were added to a 96-well plate. Reaction was initiated by the addition of 280 µl of $ABTS^{+\cdot}$. Absorbance reading was taken every 5 min at 30 °C until 2 h in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate.

The inhibition of the $ABTS^{+\cdot}$ was calculated according to the percentage of Radical Scavenging Activity (% RSA) described above (at t = 30 min.).

2.4. ORAC assay

The peroxyl radical scavenging activity of SQ was measured by $ORAC_{FL}$ assay as previously described (Prior et al., 2003). Stock solution of SQ was reconstituted in DMSO and then diluted in PBS. A stock solution of Trolox™ (TR), as reference antioxidant control, was also diluted in DMSO and diluted in PBS. The assay was carried out in 96-well plates with a final volume of 160 µL. Samples were run in triplicate. Fluorescein (48 nM) was mixed with various concentrations of SQ, standard (TR) or blank (PBS) containing at final volume 1% DMSO (v/v). Plates were incubated for 15 min at 37 °C. The assay was initiated by the addition of AAPH (100 mM) and fluorescence reading (Ex: λ_{485}/Em: λ_{520} nm) was taken every 5 min at 37 °C until 160 min in a microplate reader (TECAN GENios Plus). Final results were calculated based on the difference in the Area Under the fluorescence decay Curve (AUC) between the blank and each sample. The AUC formula was:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + ... + f_{20}/f_0.$$

Results were expressed as micromolar TR equivalents (TE) calculated using the line equation from the standard curve:

$$TE = (Y - b) / m$$

where Y is the Net AUC ($AUC_{sample} - AUC_{control}$), m is the slope and b is the Y-intercept.

2.5. Cell culture

The highly invasive MDA-MB-231 human breast cancer cells (oestrogen and progesterone receptor-negative), the minimally invasive MCF7 human breast cancer cells (oestrogen and progesterone receptor-positive), and the immortalized non-tumourigenic human breast epithelial cells (MCF10A), were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Breast tumour cells (MCF7 and MDA-MB-231) were grown as monolayer in MEM supplemented with 10% FBS, 1% Hepes Buffer, 1% Sodium Pyruvate and 1% NEAA. Human mammary epithelial cells (MCF10A) were cultivated in HuMEC Ready Medium. All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely sub-cultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments.

2.6. XTT cell proliferation assay

Cell proliferation, measured as the cellular growth of treated cells respect to untreated control, was carried out using XTT-based assay according to Scudiero et al. (1988) with some modifications. Briefly, cells were seeded into 96-well culture plates. After overnight incubation to allow cell attachment, medium was removed and replaced with fresh medium containing SQ at a series of concentrations and plates were incubated for various time points (24, 48 and 120 h) followed by a proliferation period with fresh medium until completing 6 days of incubation. At these time points, plates were incubated with XTT in RPMI without Phenol-Red for 3 h and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

$$\% \text{ viable cells} = [(A_{\text{treated cells}}) / (A_{\text{control}})] \times 100$$

where A is the difference of absorbance between optical density units ($A = OD_{450} - OD_{620}$)

All measurements were performed in triplicate and each experiment was repeated at least three times.

2.7. Cell cycle assay

Cells were seeded in 12-well culture plates and treated with different doses of SQ for 24 h. After incubation, cells were fixed with cold 70% ethanol and stored at -20 °C at least 24 h. Subsequent to propidium iodide labelling (PI/RNase Staining Buffer), cells were analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). FlowJo program (v5.7.2) was used to calculate the percentage of the cells in G_0/G_1, S and G_2/M phases. Each experiment was repeated at least three independent times.

2.8. Analysis of apoptosis

Percentage of apoptotic cell was determined using a double staining assay with FITC-conjugated Annexin V and propidium iodide (PI). Briefly, after cell exposure with SQ for 24 h, cells were harvested, washed twice in cold PBS and resuspended in 100 L Annexin Binding Buffer. Cells were stained with 5 L Annexin V-FITC and 1 L PI solution, gently vortex and incubated for 15 min at room temperature in the dark before flow cytometric analysis. As a positive control, cells were treated with 1 M camptothecin (CPT). Each experiment was repeated at least three independent times.

2.9. Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) level were measured after 24 h of treatment with different doses of SQ, or TOC as a positive control, using the cell-permeable fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described (Wang and Joseph, 1999) with some modifications. In brief, after incubation, cells were washed two times with Hank's buffered salt solution (HBSS) and incubated with freshly DCFH-DA (100 M) during 30 min at 37 °C and 5% CO_2. Cells were then washed twice and read in a plate reader for 30 min (Ex. $_{.485}$/Em. $_{.535,}$ Gain 60). The intracellular ROS level percentage was calculated as follow:

$$F = [(Ft_{30}-Ft_0)/Ft_0] \times 100$$

where Ft_0 is the fluorescence at t=0 min. and Ft_{30} the fluorescence at t=30 min.

It has been described that oxidative stress increases on culture cells by the addition of H_2O_2 (Lee et al., 2006). Therefore, in order to evaluate the protective capacity of SQ against the induced oxidative stress, H_2O_2 at 500 M was added 30 min before the fluorescence quantification.

All tests were run in triplicate for each experimental condition and each experiment was repeated at least three times. All experiments were conducted using iron-free mediums (MEM and HuMEC).

2.10. Alkaline Single-cell gel electrophoresis (comet assay)

24 h treated cells with SQ into 12-well culture plates were scrapped, washed twice (300xg 10 min, 4 °C) with cold 1xPBS (Ca^{2+}/Mg^{2+} free) and then resuspended in 1 ml of cold 1xPBS. In order to evaluate SQ protection against oxidative DNA damage, cell suspensions were exposed for 10 min to 50 µM H_2O_2 at 4 °C. After that, cells were washed twice and frozen in FBS-DMSO (90:10, v/v) at -80 °C until comet assay procedure.

DNA single strand break by alkaline microgel electrophoresis was performed according to Singh et al. (1988) with some modifications. Cells were thawed in bath at 37 °C, centrifuged (300xg 10 min, 4 °C) in cold MEM 25% FBS and adjust to $1.65x10^5$ cells/ml in cold 1xPBS. Cells were suspended in melted and cooled at 40 °C low melting point agarose (LMA). Cell suspensions (50 µL) were spread over sample area of pre-warmed 1% normal melting point agarose (NMA) precoated CometSlide™ slides (Trevigen, Inc). After 15 min at 4 °C in the dark, slides were immersed in cold Lysis Solution (Trevigen, Inc) at 4 °C for 30 min to dissolve lipids and proteins. In order to separate the two DNA strands, slides were then immersed in freshly Alkaline Solution (pH > 13) for 30 min at room temperature in the dark. Electrophoresis was performed in a refrigerated black electrophoresis tank containing cold Alkaline Electrophoresis Solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 25V (1V/cm) and 300 mA for 40 min. The slides were washed twice with distilled water for 10 min and neutralized with 10 mM Tris-HCl, pH 7.5 for 5 min, followed by immersion in 70% ethanol for 5 min and air-dried overnight at room temperature. Slides were stained with Sybr green before scoring.

2.11. Slide scoring and analysis

DNA strand breaks were examined in a fluorescence microscope (Zeiss Axiovert 200) equipped with Luca EMCCD camera (Andor Technology, Belfast, UK) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). Fifty cell images were randomly characterized per sample using x20 magnifications. Relative fluorescence between head and tail through Olive tail moment (Olive_TM) was used to determine DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

$$\text{Olive\_TM} = [(\text{Tail (mean)} - \text{Head (mean)}) \times \text{Tail (\% DNA)}] / 100.$$

2.12. Statistical analysis

The results are presented as mean (± SEM), except for cell proliferation. For this assay, results are presented as mean (± SD). Results are expressed as percentage respect to the control, which was considered to be 100%. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test. Values of $p < 0.05$ were considered significant. STATGRAPHICS Plus 5.1 statistical software (Rockville, MA, USA) was used for the statistical analysis.

3. Results

3.1. Antioxidant activity

Radical scavenging capacity was determined using DPPH, ABTS and ORAC assays.

The antiradical activity of SQ by the scavenging activity on DPPH radical assay (Fig. 1A) or on ABTS cationic radical (Fig. 2A) indicates that SQ does not possess antiradical activity up to

10 mole ratio or up to 800 μM respectively. TOC or TR were used respectively as antioxidant standard control for DPPH (Fig. 1B) or ABTS (Fig. 2B) assays.

Peroxyl radical scavenging activity of SQ, measured by $ORAC_{FL}$ assay, shows the absence of protective effect against AAPH-induced peroxyl radical (Fig 3A) compared to TR antiradical activity (Fig. 3B).

3.2. Effect of SQ on cell proliferation

To observe the effect of SQ on both, human breast cancer cell lines (MCF7, MDA-MB-231) and on immortalized non-tumourigenic human mammary epithelial cell line (MCF10A), cells were treated by various concentrations of SQ ranging from 3.12 to 50 M for 24, 48 and 120 h. Results showed that SQ had no significant effect on the cell proliferation rate on both, MCF7 and MCF10A cells (Fig. 4A, 4C). Unexpectedly, a slight increase on MDA-MB-231 cell proliferation was observed, although it was not statistically significant (Fig. 4B).

3.3. Effect of SQ on cell cycle

Flow cytometry assay after PI staining allows cell cycle analysis. MCF7, MDA-MB-231 and MCF10A cells were treated with 12.5, 50 and 200 M of SQ to evaluate the interference with the cell cycle. The results revealed that after 24 h of SQ treatment, no significant effect was observed on the cell cycle on any one of the three cell lines tested (data not shown).

3.4. Effect of SQ on apoptosis

The extent of apoptosis was assessed by flow cytometry analysis following 24 h cells exposure to different doses of SQ (12.5, 50 to 200 M). Apoptosis was detected using Annexin-V-FITC (AV) and PI labelling. The percentage of apoptotic cells was calculated as the sum of early plus late apoptotic cells and referred to the whole cells. Flow cytometric analysis revealed that treatment with SQ for 24 h did not induce apoptosis neither in human breast epithelial cells

(MCF10A) nor in human breast cancer cells (MCF7, MDA-MB-231) when compared to the control (data not shown).

3.5. Effect of SQ on intracellular ROS production

Measurement of intracellular ROS level using DCFH-DA probe, showed a significant and dose-dependent manner decrease of ROS level on MCF10A treated cells after 24 h with increasing concentrations of SQ from 3.12 to 50 M. At the highest concentration (50 M), SQ reduced in more than 50% the ROS production compared to the control without treatment. Interestingly, SQ did not change intracellular ROS level on both human breast cancer cells tested (MCF7, MDA-MB-231) (Fig. 5A).

In the present study, TOC, used as antioxidant reference compound, exhibit a significant decrease of ROS level, getting over than 50% reduction compared with untreated cells on each one of the cell lines assayed (MCF10A, MCF7 and MDA-MB-231) (Fig. 5B).

To induce an intracellular oxidative stress, H_2O_2 was added before fluorescence measurement. Effectively, H_2O_2 induces oxidative injury on MCF7, MDA-MB-231 and MCF-10A cells *in vitro* (data not shown). In order to investigate the *in vitro* preventive effect of SQ against H_2O_2 oxidative injury, intracellular ROS level was measured on cells previously treated with SQ for 24 h at increasing concentrations from 3.12 to 50 M. Interestingly, MCF10A epithelial cells showed a significant decrease in intracellular ROS level in a dose-dependent manner up to 60% reduction whereas SQ did not prevent oxidative stress injury on MCF7 and MDA-MB-231 breast tumour cells (Fig. 6A). In contrast, TOC was able to reduce oxidative injury from the lowest concentration (3.12 μM) on both, tumoral and epithelial cells (Fig. 6B). Thus, SQ is more effective reducing oxidative injury than TOC on MCF10A epithelial cells.

3.6. Preventive effect of SQ on oxidative DNA damage

We measured the preventive effect of SQ on H_2O_2-induced DNA damage in MCF10A, MCF7 and MDA-MB-231 cells using alkaline single-cell gel electrophoresis (comet assay). The potential of SQ to reduce oxidative DNA damage was expressed as Olive_TM. Olive_TM

incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail) (Sing et al., 1988).

Pre-incubation of MCF10A cells with increasing concentrations of SQ for 24 h exerted a significant preventive dose-dependent effect over H_2O_2-induced DNA damage (P<0.001) (Fig. 7A). However, SQ did not show any preventive effect against the formation of single-strand breaks on MCF7 and MDA-MB-231 tumour cells. Definitely, an increase in the oxidative DNA damage was observed on MDA-MB-231 cell line, although it was no statistically significant (P=0.23) (Fig. 7B).

4. Discussion

Case control studies have shown an inverse correlation between olive oil consumption and the incidence of breast cancer (Escrich et al., 2006; Owen et al., 2000a). It has been suggested that the lower risk of breast cancer associated with olive oil consumption may be due to the presence of SQ (Newmark, 1997). SQ is a minor compound present in virgin olive oil in quantities as high as 13000 mg/kg (Allouche et al., 2007). The average SQ daily dietary intake in Mediterranean countries is in the range of 200-400 mg/day (Sotiroudis and Kyrtopoulos, 2008).

Experiments in rodents suggest the antitumour activity of SQ against skin, colon, sarcoma and lung cancer (Murakoshi et al., 1992; Ohkuma et al., 1983; Rao et al., 1998; Smith et al., 1998). It has also been shown that SQ inhibits the *in vitro* growth of neuroblastoma cells (Das et al., 2003). The mechanism proposed to explain the activity of SQ is based on its strong inhibitory action of beta-hydroxy-beta-methylglutaryl-CoA reductase catalytic activity *in vivo* which affects Ras p21 farnesylation, signal transduction and cellular proliferation (Newmark, 1997). Nevertheless, very little is known about the effect of SQ on human breast tumourigenesis.

Our data suggest that SQ treatment did not exert any significant influence on MCF10A, MCF7 and MDA-MB-231 cell proliferation assay despite we observed a slight increase on MDA-MB-231 cell proliferation. Das et al. (2008) have hypothesized that SQ might support the growth of a small fraction of tumour stem cell-like cells having a very high tumourigenic

capacity after cisplatin treatment. In addition, it has been reported that at low-doses, certain individual antioxidants can stimulate the growth of some types of cancer. For example, vitamin C was found to stimulate the growth of both human parotid carcinoma cells and human leukemic cells *in vitro* (Prasad et al., 2001), whereas vitamin E enhanced the growth of different prostate cancer cell lines (Bureyko et al., 2009). Beta-carotene, an antioxidant structurally similar to SQ, increased the incidence of lung cancer among male heavy cigarette smokers (Paolini et al., 2003). Future works are needed to clarify the mechanisms of stimulation of cancer cell growth by some antioxidants.

In the present study, the effect of SQ on cell cycle profile and cell apoptosis was also studied. Data showed that the incubation of MCF10A, MCF7 and MDA-MB-231 cells for 24 h with SQ did not alter the cell cycle parameters or induced cell apoptosis. Overall, these results suggest that SQ don't induce breast tumour cell death and may be ineffective once breast tumour is established.

SQ has long been considered as an antioxidant exerting anticarcinogenic activity by enhancing cellular antioxidant status (Hashim et al., 2005; Murakoshi et al., 1992; Smith, 2000), but very little is known about SQ scavenging capacity. Our results showed that SQ did not exert antioxidant activity either against DPPH stable radical, ABTS cation radical or APPH-induced peroxyl radicals even at high concentration (10.00 mole ratio, 800 μM and 400 μM respectively) (Fig. 1, Fig. 2 and Fig. 3). Concomitantly, it has been reported that SQ reacts very poorly with DPPH (EC50 at 980 mole ratio) (Psomiadou and Tsimidou, 1999), and has very low ORAC value (0.00062) suggesting that SQ possesses an extremely low antioxidant activity (Tikekar et al., 2008). The absence of ABTS scavenging capacity of SQ has not been described previously. Otherwise, Kohno et al. (1995) described that SQ is a singlet oxygen quencher protecting the skin from UV radiation. SQ is permeable to plasmatic membrane and so it can be moved inside the bilayer-lipidic membrane acting as a quencher barrier for singlet oxygen and consequently preventing intracellular $O_2^{\cdot}$ oxidative damage. Differential SQ permeability among tumour and non-tumour mammary cells could be a possible mechanism but nowadays not conclusive results have been described.

SQ has been found to have protective activity against several carcinogens, including azoxymethane-induced colon cancer (Rao et al., 1998) and nicotine-derived nitrosaminoketone induced lung carcinogenesis (Smith et al., 1998). In the present study, antioxidant potential of SQ evaluated by DCFH-DA assay indicated that SQ has differential antioxidant activity on

human breast cells since it significantly decreased in a dose-dependent manner intracellular ROS level on MCF10A cells (57.71% at 50 μM) but not on MCF7 or MDA-MB-231 cells (104.87% and 99.92% at 50 μM, respectively). This selective effect of SQ was also described by Das et al. (2008), although on other cell types. These authors found that SQ decreased ROS level on bone marrow cells but not on neuroblastoma cells (SK-N-BE tumour cell line). On the other hand, the well-known antioxidant TOC, used as reference in the present study, did not show this selective effect reducing the oxidative stress by 58.84%, 53.78% and 64.20% at 50 μM respectively on MCF10A, MCF7 and MDA-MB-231 cells.

Consequently, our results suggest that breast cancer cells as well as breast epithelial cells have the same response to TOC treatment, but SQ acts as an antioxidant only on mammary epithelial cells. Taking into account that the mechanism of such selective antioxidant sensitivity is unknown, we would have to considerer the following possibilities: (i) it is possible that SQ selectively increases glutathione (GSH) level on normal but not on breast cancer cells, a phenomenon described as the "GSH paradox" (Das et al., 2003), (ii) it might be related to the difference in the cellular uptake and accumulation, or the status of the mevalonate pathway (Das et al., 2008), (iii) it might be related to the differential regulation of antioxidant system on normal *versus* tumour cells (Klauning and Kamendulis, 2004). Tumour cells may be under a continued environment of oxidative stress, where antioxidant may acts as a pro-oxidant. In fact, in the present study we found that breast cancer cells have higher intracellular ROS level than non-tumourigenic mammary epithelial cells (data not shown). Further studies should be done to respond these topics.

Oxidative stress may result in an increase of oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant system. It is known that chronic increase in ROS and oxidant stress may induce cancer (Ambrosone, 2000). Reactive oxygen species can damage different cellular macromolecules, including DNA, which is directly responsible for carcinogenesis. Thus, it has been shown that neoplastic transformation is associated with an increase in the basal oxidant level. In fact, serum markers for oxidative DNA damage have been shown to increase in women diagnosed with breast cancer (Musarrat et al., 1996). Considering that oxidative stress is involved in the pathophysiology of all cancers (Visioli et al., 2004), prevention of oxidative stress on mammary cells could be a suitable way to prevent breast cancer development.

In the present work, we found that H_2O_2-induced oxidative injury increases ROS level on both, human mammary epithelial and breast cancer cells as measured by the DCFH-DA assay (data not shown). Interestingly, SQ was able to prevent in a dose-dependent manner the oxidative injury induced on human breast epithelial cells but not on human breast cancer cells. It has been postulated that neoplastic transformation is associated with an increase in the basal level of oxidant stress; therefore, it may be presumed that SQ might help to prevent human breast cancer by reducing oxidative stress on mammary epithelial cells.

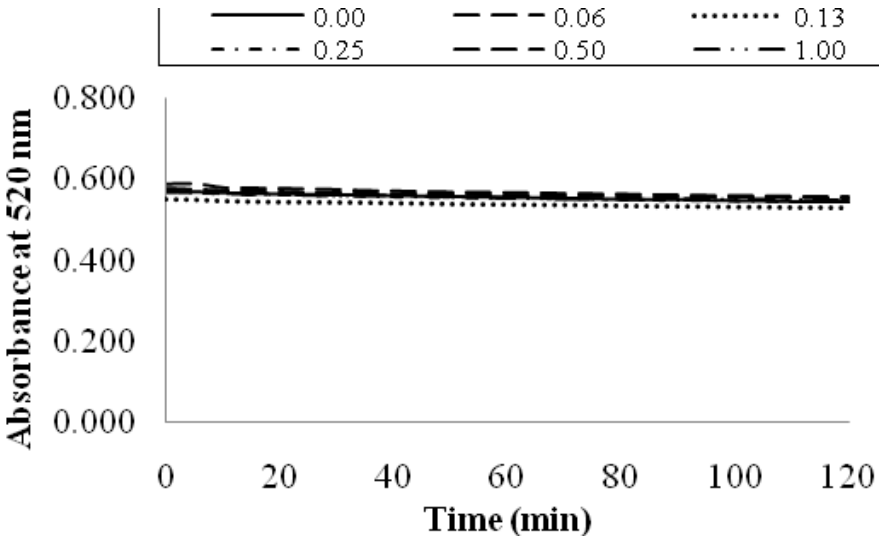
Comet assay provides information about DNA damage through quantification of single-strand breaks. We used this technique to determine the protective effect of SQ on oxidative DNA damage induced by H_2O_2 oxidative injury. As observed in DCFH-DA assay, SQ exhibited selective activity according to the cell line, reducing oxidative DNA damage in a dose-dependent manner on MCF10A mammary epithelial cells, whereas oxidative injury was maintained on MCF7 or even slightly increased on MDA-MB-231 breast cancer cells after SQ treatment.

Therefore, these results suggest that SQ might be helpful in human breast cancer prevention based on two hypotheses, (i) it reduces the oxidative stress by decreasing ROS level on mammary epithelial cells, and (ii) it selectively protects against oxidative DNA damage in mammary epithelial cells. In addition, these findings could support the hypothesis that high SQ intake could contribute to the lower incidence of breast cancer in Mediterranean populations. It is important to note that adipose tissue contains exceptionally high concentrations of SQ than other tissues (Tilvis et al., 1982). Considering that mammary gland contains adipose tissue, it may be presumed that SQ level could also be high in breast tissues.

In summary, we found that mammary epithelial cells and breast tumour cells differed in their responses to SQ treatment. Our results showed that SQ reduces *in vitro* ROS level and protects against oxidative DNA damage on human mammary epithelial cell line but does not on breast tumour cell lines. Therefore SQ, presents at high concentration in virgin olive oil, might contribute to the preventive effect of the olive oil on human breast cancer inhibiting oxidative stress. Moreover, our *in vitro* results suggest that SQ might be important in the prevention of human breast cancer, but ineffective once breast tumour is established. Nevertheless, extreme caution should be applied to extrapolate *in vitro* results to potential human effects. Further studies are needed to confirm both: the *in vivo* protective capacity of SQ and, the differential mechanism of action on normal *versus* breast cancer cells suggested in the present study.

Figure 1

A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0.06
0.13
0.25
0.50
1.00
Absorbance at 520 nm
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0.600
0.400
0.200
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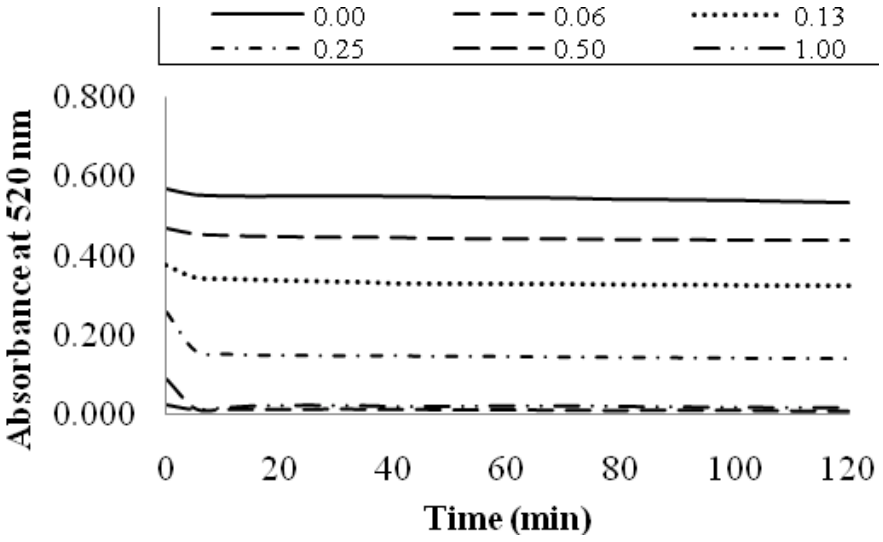
20
40
60
80
100
120
Time (min)

B


0.00
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1.00
Absorbance at 520 nm
0.800
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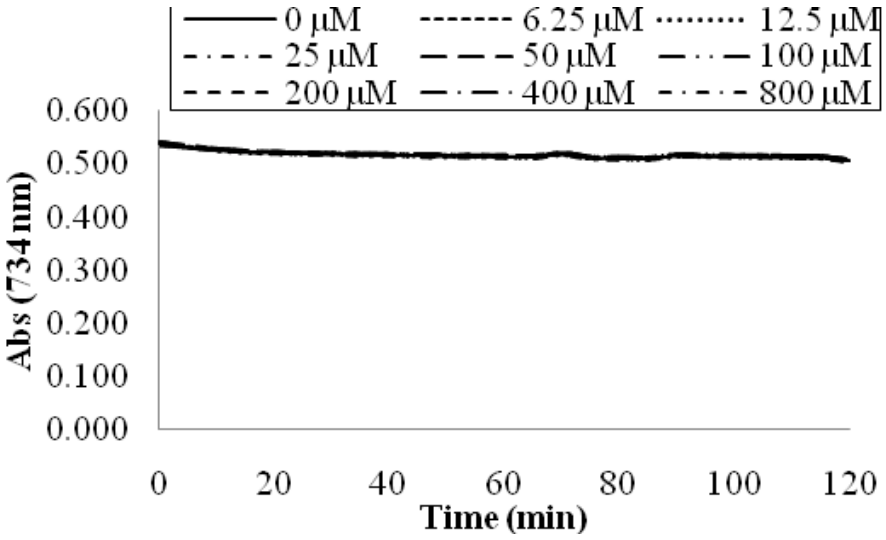
0.200
0.000
0
20
40
60
80
100
120
Time (min)

Figure 2

A


0 µM
6.25 µM
12.5 µM
25 µM
50 µM
100 µM
200 µM
400 µM
800 µM
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Abs (734 nm)
0
20
40
60
80
100
120
Time (min)

B

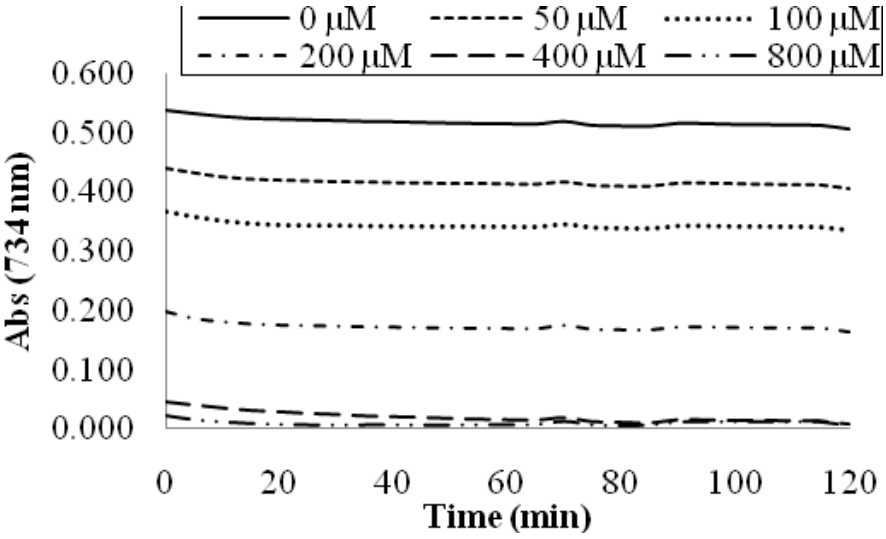
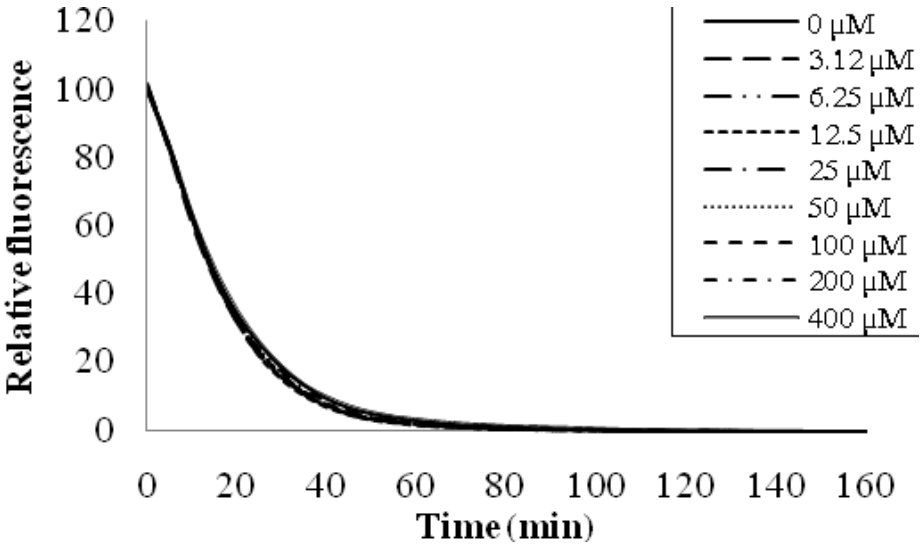

0 µM
50 µM
100 µM
200 µM
400 µM
800 µM
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Abs (734 nm)
0
20
40
60
80
100
120
Time (min)

Figure 3

A


Relative fluorescence
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Time (min)
0 µM
3.12 µM
6.25 µM
12.5 µM
25 µM
50 µM
100 µM
200 µM
400 µM

B

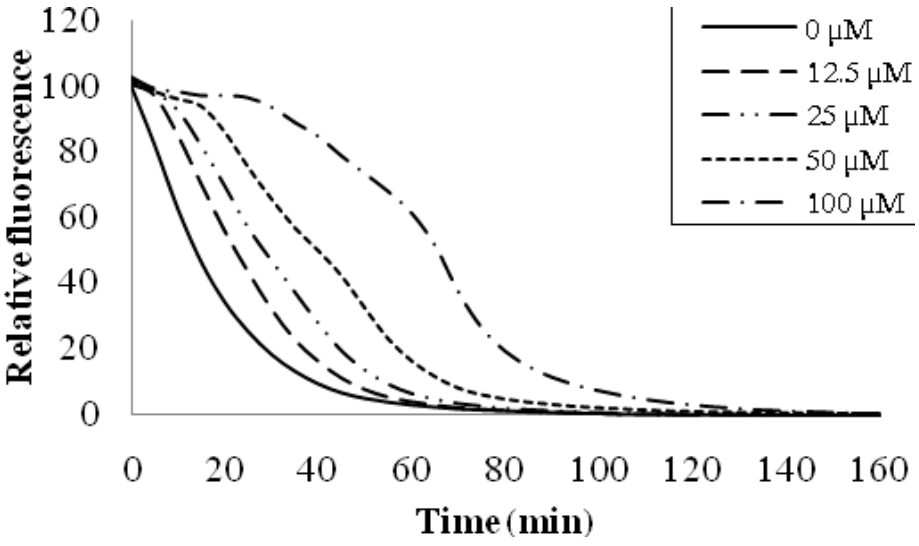
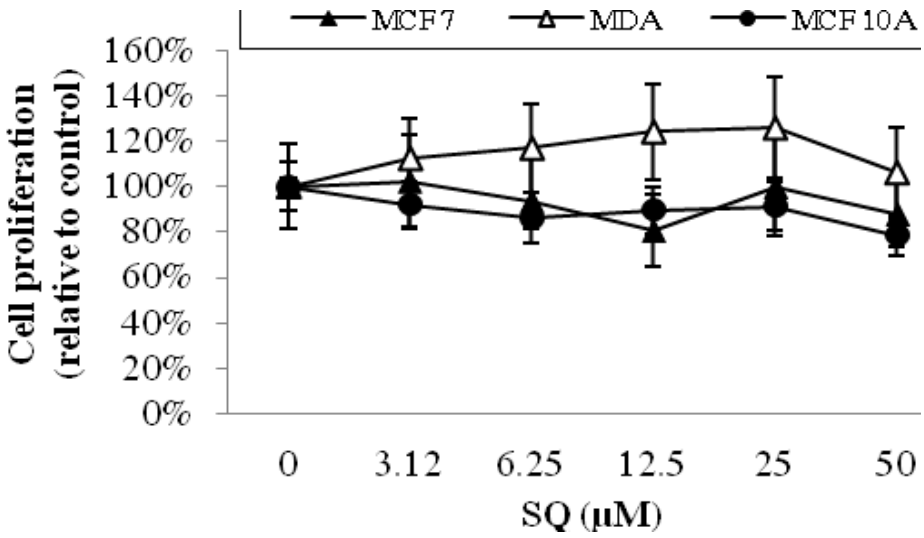
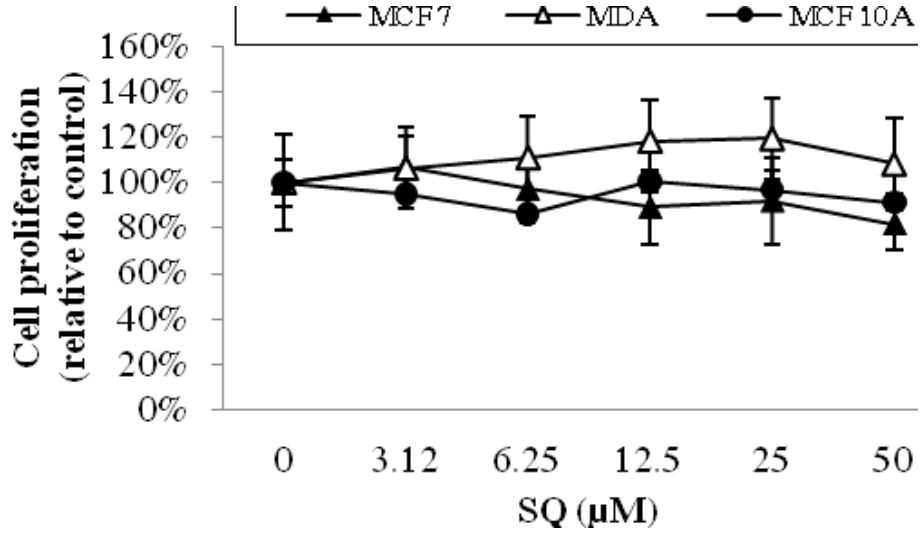

Relative fluorescence
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Time (min)
0 µM
12.5 µM
25 µM
50 µM
100 µM

Figure 4

A


MCF7
MDA
MCF10A
Cell proliferation (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
3.12
6.25
12.5
25
50
SQ (µM)

B


MCF7
MDA
MCF10A
Cell proliferation (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
3.12
6.25
12.5
25
50
SQ (µM)

C

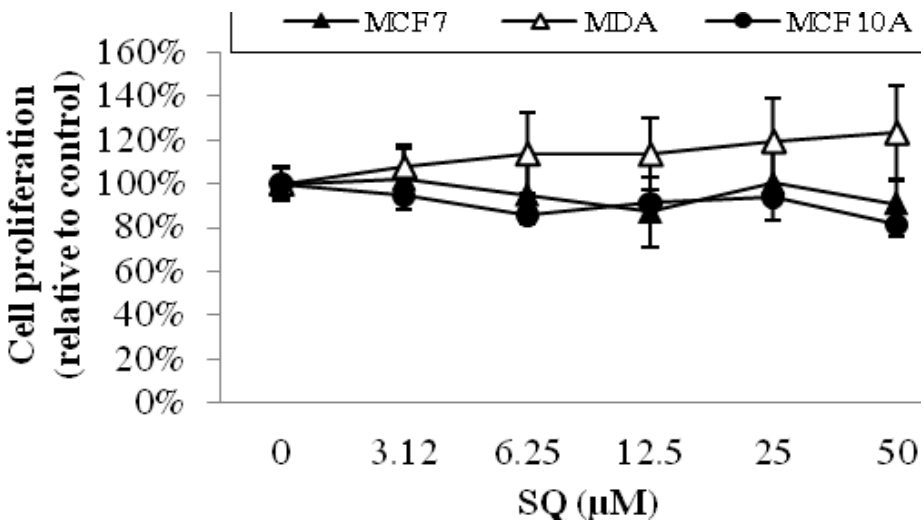
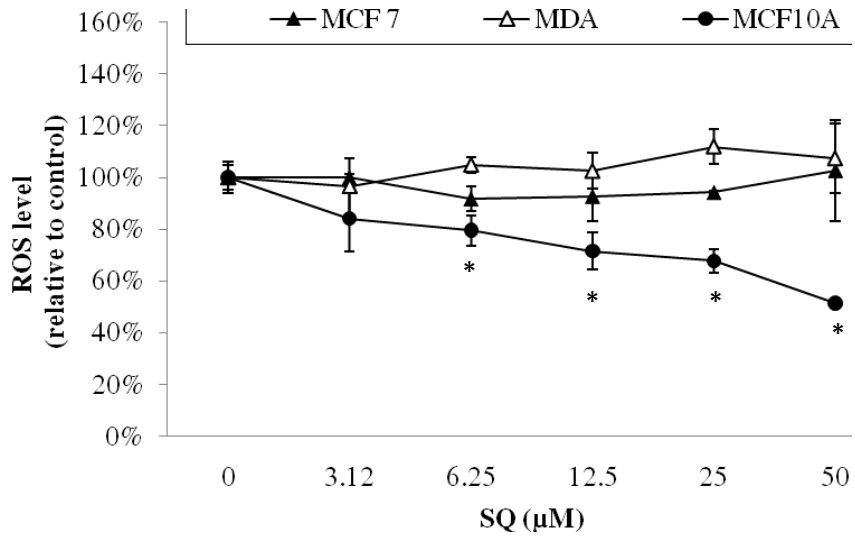

MCF7
MDA
MCF10A
Cell proliferation (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
3.12
6.25
12.5
25
50
SQ (µM)

Figure 5

A


MCF 7
MDA
MCF10A
ROS level (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
*
*
*
*
0
3.12
6.25
12.5
25
50
SQ (µM)

B

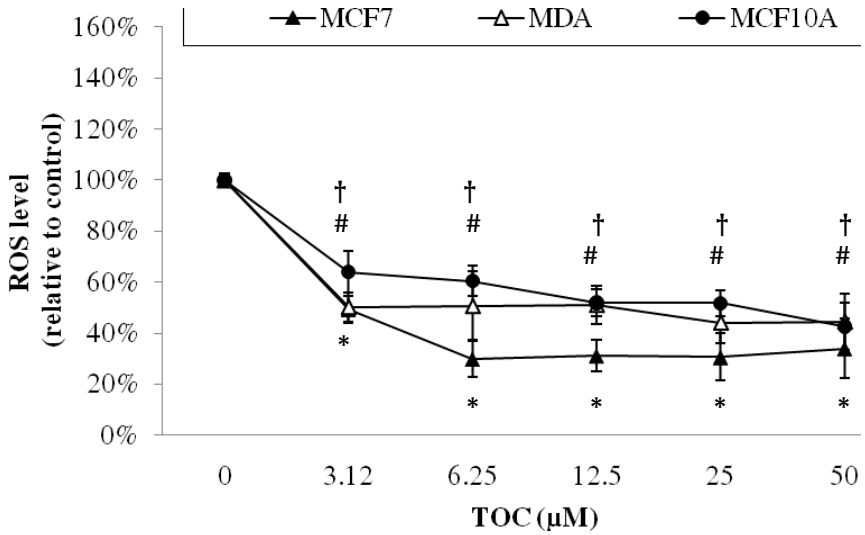
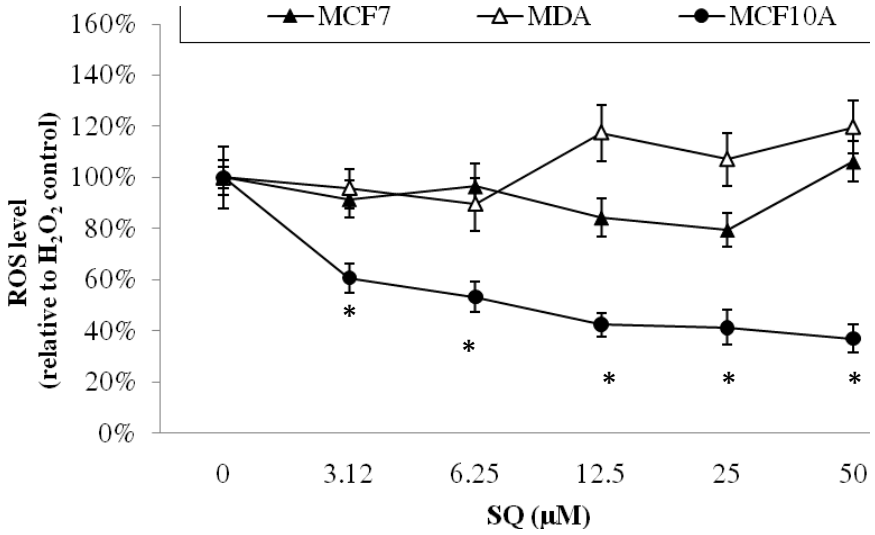

MCF7
MDA
MCF10A
160%
140%
120%
100%
80%
60%
40%
20%
0%
ROS level
(relative to control)
0
3.12
6.25
12.5
25
50
TOC (µM)

Figure 6

A


MCF7
MDA
MCF10A
160%
140%
120%
100%
80%
60%
40%
20%
0%
ROS level
(relative to H_2O_2 control)
0
3.12
6.25
12.5
25
50
SQ (µM)

B

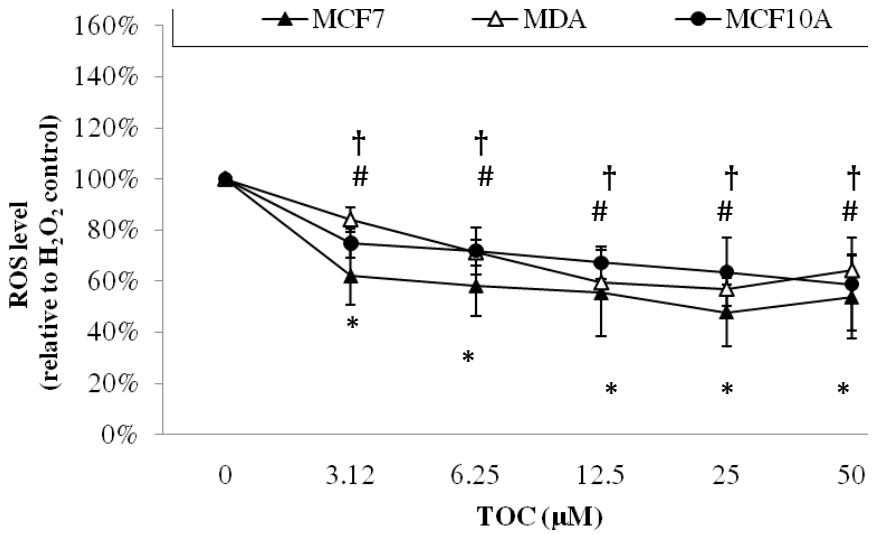
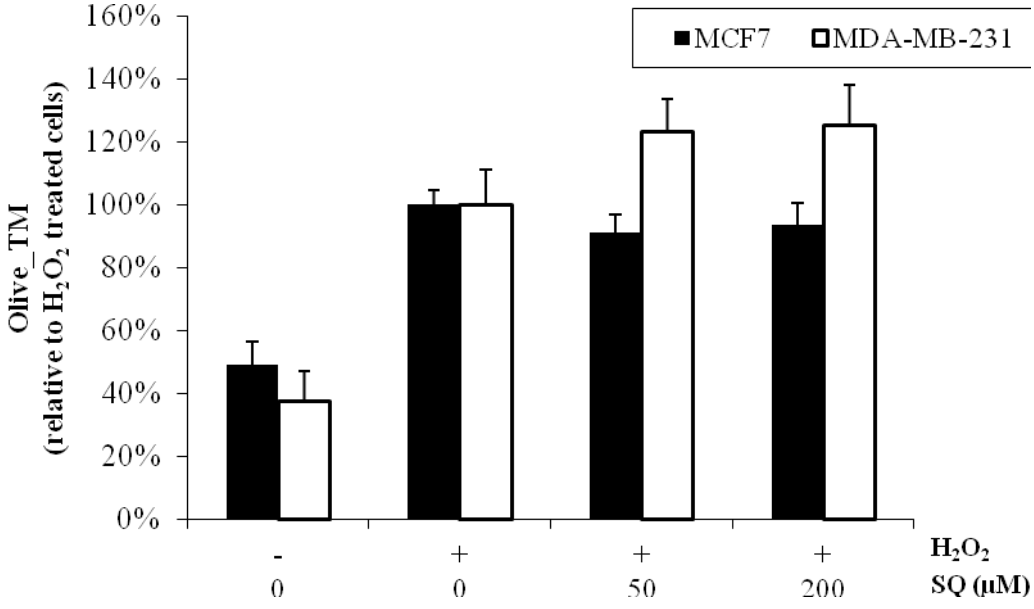
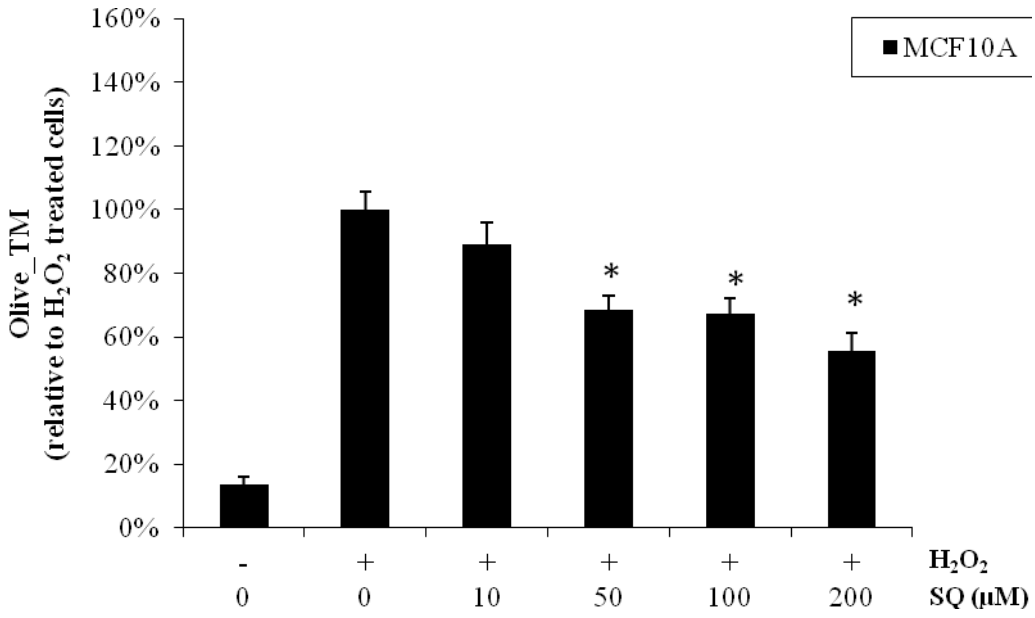

MCF7
MDA
MCF10A
160%
140%
120%
100%
80%
60%
40%
20%
0%
ROS level
(relative to H_2O_2 control)
0
3.12
6.25
12.5
25
50
TOC (µM)

Figure 7

A


MCF7
MDA-MB-231
160%
140%
120%
100%
80%
60%
40%
20%
0%
Olive_TM
(relative to H_2O_2 treated cells)
-
+
+
+
H_2O_2
0
0
50
200
SQ (µM)

B



Legends:

Fig 1: Radical scavenging activity was measured through the reduction of the DPPH radical (100 µM) by ethanolic solutions of squalene (SQ) (A) and α-tocopherol (TOC) (B) for the period of 120 min. Mole ratio [mole antioxidant/mole DPPH] assayed was between 0.06 and 1. Relative Radical Scavenging Activity (RSA) of SQ at 50 min was ≤ 5% even at higher ratios (2.50, 5.00 and 10.00) (data not shown) while 50% RSA of TR was at 0.16 mole ratio.

Fig. 2. ABTS radical cation ($ABTS^{+\cdot}$) antioxidant activity of ethanolic solutions of squalene (SQ) (A) or Trolox™ (TR) (B) up to 800 µM were measured by decolorization of $ABTS^{+\cdot}$ for 120 min. Relative Radical Scavenging Activity (RSA) for every assayed concentrations of SQ at 30 min was < 4% while 50% RSA of TR was at 143.57 µM.

Fig. 3. Peroxyl antiradical activity was performed by $ORAC_{FL}$ assay on a range of concentrations of squalene (SQ) from 3.12 to 400 µM (A) or Trolox™ (TR) from 12.5 to 100 µM

(B) for 160 min. Results show how SQ fails to reduce the AAPH-induced peroxyl radical at any one of the assayed concentrations compared with TR. TE for any one of the SQ concentrations assayed was close to 0 indicating the absence of peroxyl scavenging activity (data not shown).

Fig. 4. *In vitro* cell proliferation of squalene (SQ) treatment on MCF7, MDA-MB-231 and MCF10A cell lines was determined by colorimetric XTT-based assay at 24 h (A), 48 h (B) and 120 h (C). Although MDA-MB-231 showed a slight increase in cell proliferation, no difference at $p < 0.05$ was detected in any cell line. Higher concentrations of SQ up to 400 μM showed similar effects (data not shown). Values represent the mean ± SD of three independent experiments and are expressed as a percentage of the control, which was considered to be 100%.

Fig. 5. Intracellular ROS level was measured by DCFH-DA probe on MCF7, MDA-MB-231 and MCF10A cell lines after treatment with squalene SQ (A) or α–tocopherol TOC (B) during 24 h. SQ significantly decrease intracellular ROS level in a dose-dependent manner in MCF10A cells but not in MCF7, MDA-MB-231 cells. TOC significantly decrease the intracellular ROS level in a dose-dependent manner in both, breast cancer and mammary epithelial cell lines. Results are displayed as mean ± SEM of three independent experiments. Significant differences were compared with the control, which was considered to be 100%, at $p < 0.05$ († MCF7, # MDA-MB-231, * MCF10A) using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

Fig. 6. Capacity of squalene (SQ) (A) or α-tocopherol (TOC), as antioxidant control (B), to reduce an oxidative burst induced by addition of H_2O_2 (500 μM) on MCF7, MDA-MB-231 or MCF10A cell lines was measured by DCFH-DA probe. SQ prevents in a dose-dependent manner the oxidative injury induced on MCF10A cells, but preventive effect was not observed on MCF7 and MDA-MB-231 cells. Interestingly, TOC reduce oxidative injury on the three cell lines significantly since the lowest concentration (3.12 μM). Results are expressed as mean ± SEM of three independent assays. Significant differences were compared with the positive control (treated with H_2O_2), which was considered to be 100%, at $p < 0.05$ († MCF7, # MDA-MB-231, * MCF10A) using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

Fig. 7. Comet assay was performed in order to determine the preventive effect of squalene (SQ) in DNA oxidative damage on MCF10 cell line (A) or MCF7 and MBA-MB-231 cell lines (B). SQ reduces single-strand breaks in a dose-dependent manner on MCF10A cells, whereas no preventive effect was observed on MCF7 and MBA-MB-231 cells. Olive_TM values are represented by mean ± SEM of three independent assays. Significant differences were compared with the positive control (treated only with H_2O_2), which was considered to be 100%, at * $p < 0.05$ using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells

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Received: 27 July 2011; in revised form: 8 September 2011 / Accepted: 14 September 2011 / Published: 13 October 2011

Abstract: Over recent years, several studies have related olive oil ingestion to a low incidence of several diseases, including breast cancer. Hydroxytyrosol and tyrosol are two of the major phenols present in virgin olive oils. Despite the fact that they have been linked to cancer prevention, there is no evidence that clarifies their effect in human breast tumor and non-tumor cells. In the present work, we present hydroxytyrosol and tyrosol's effects in human breast cell lines. Our results show that hydroxytyrosol acts as a more efficient free radical scavenger than tyrosol, but both fail to affect cell proliferation rates, cell cycle profile or cell apoptosis in human mammary epithelial cells (MCF10A) or breast cancer cells (MDA-MB-231

and MCF7). We found that hydroxytyrosol decreases the intracellular reactive oxygen species (ROS) level in MCF10A cells but not in MCF7 or MDA-MB-231 cells while very high amounts of tyrosol is needed to decrease the ROS level in MCF10A cells. Interestingly, hydroxytyrosol prevents oxidative DNA damage in the three breast cell lines. Therefore, our data suggest that simple phenol hydroxytyrosol could contribute to a lower incidence of breast cancer in populations that consume virgin olive oil due to its antioxidant activity and its protection against oxidative DNA damage in mammary cells.

Keywords: breast cancer; Mediterranean diet; olive oil minor compounds; hydroxytyrosol; tyrosol; phenols; oxidative stress; reactive oxygen species; DNA damage

1. Introduction

Olive oil is the major source of fats in the Mediterranean diet and is considered to be responsible for the health benefits associated with this diet. In fact, it has been demonstrated that people who consume virgin olive oil (VOO) present a lower incidence of several cancers, including breast cancer [1]. This effect has previously been attributed to the high content of monounsaturated fatty acids. However, more recently, the importance of the minor constituents of olive oil has been considered [2]. Over the last five decades, several publications have firmly established that ingestion of small quantities of certain compounds isolated from plants can lower the risk of cancer in mammals exposed to carcinogens, including polyphenols [3].

VOO contains relatively high amounts of minor compounds compared to other oils (refined olive oil or seed oils). Among these, phenolic compounds are present at levels between 200 and 1500 mg/kg [4] depending on the olive tree variety, climatic and agronomic conditions, degree of maturation at harvest, and the manufacturing process [4]. At present, there are many studies reporting biological activities *in vitro*, *in vivo* and in clinical assays of phenolic compounds naturally present in VOO. Between them, anti-inflammatory, cardioprotective antioxidant and chemopreventive effects in breast and other types of cancers have been defined [5]. The major phenols identified in olive oils include the simple phenols hydroxytyrosol (HT) and tyrosol (TY), secoiridoids and lignans [2]. The concentration of TY is always higher than of HT [6]. Hydrolysis of secoiridoid during olive oil storage results in the formation of HT and TY [7].

It has been well established that HT is a potent antioxidant because of its marked antioxidant activity, its ability to scavenge oxygen and nitrogen free radicals, to inhibit Low Density Lipoprotein (LDL) oxidation, platelet aggregation and endothelial cell activation and its protection against DNA damage [2,8]. HT was able to reduce the synthesis of prostaglandin E2 blocking the transcription of COX-2 and 5-lipooxygenase, thereby reducing the chronic influence associated with diseases such as cancer [9]. TY has been described as exerting a weak antioxidant activity, although it is able to scavenge peroxynitrite and superoxide radicals, inhibit LDL oxidation in Caco2 cells and inhibit LPS-induced cytokines release from human monocytes [10,11].

It has been suggested that HT and TY compounds might have preventive activity against breast cancer, but, at present, the exact role played by these phenols in breast cancer prevention is still unknown. In this sense, despite epidemiological evidence, *in vitro* experiments have not been conducted to check if there are different effects of the simple phenols HT and TY between human breast cancer cells and human breast non-cancer cells.

The present study attempts to provide new insights into the antioxidant capacity of HT and TY and the *in vitro* effects on proliferation, cell cycle progression, apoptosis, reactive oxygen species (ROS) production and oxidative DNA damage in the human breast epithelial MCF10A cell line and the human breast MCF7 and MDA-MB-231 cancer cell lines.

2. Experimental Section

2.1. Chemicals and Materials

The following were purchased from Sigma-Aldrich Co. (St Louis, MO, USA): Hepes Buffer; Sodium Pyruvate; Non-Essential Amino Acids mixture 100× (NEAA); 2′,7′-dichlorofluorescein diacetate (DCFH-DA); Dimethyl sulfoxide (DMSO); 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT sodium salt) purity ≥90%; *N*-Methylphenazonium methyl sulfate (PMS) purity ~98%; 2-hydroxyphenyl ethanol (Tyrosol, CAS 501-94-0 (TY)) purity 98%; DL-all-rac-α-Tocopherol (Vitamin E, CAS 10191-41-0 (TOC)) purity ≥96%; 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity ≥97%; 2,2′-Azobis (2-methylpropionamidine) dihydrochloride

(AAPH) purity ~97%; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) purity ~90%, (*S*)-(+)-camptothecin (CAS 7689-03-4 (CPT)) purity ~95%; 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt tablets (CAS 30931-67-0 (ABTS)); PBS; HBSS. 2-(3,4-dihydroxyphenyl) ethanol (Hydroxytyrosol, CAS 10597-60-1 (HY)) purity ≥98% was obtained from Cayman Chemical (Ann Arbor, MI, USA). Minimum essential medium with Eagle's salts (MEM), Fetal Bovine Serum (FBS) and Phenol-Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express, HuMEC Ready Medium kit and Fluorescein (FL) were obtained from Invitrogen (Eugene, OR, USA). $K_2S_2O_8$ (CAS 7727-21-1) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Culture plates were obtained from NUNC™ (Roskilde, Denmark). The PI/RNase Staining Buffer kit, FITC-conjugated Annexin V and Binding Buffer were obtained from BD Biosciences Pharmigen (San Diego, CA, USA). The Comet assay kit was obtained from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD, USA).

2.2. DPPH Assay

The antioxidant activity of HT and TY against the stable radical DPPH was measured as previously reported [12] with some modifications. Briefly, 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of HT or TY in 96-well plates at 0.06, 0.13, 0.25, 0.5 and 1 (moles of antioxidant/moles of DPPH). (±)-α-tocopherol (TOC) was used as a positive control and a sample without antioxidant was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min for 2 h in a microplate reader (TECAN, GENios Plus). Measurements were performed in triplicate.

The inhibition of the DPPH radical was calculated according to the following percentage of Radical Scavenging Activity (% RSA) formula:

$$\% \text{ RSA} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 50 min.

2.3. ABTS Assay

ABTS cation radical scavenging activity was determined using a previously reported procedure [13]. ABTS radicals ($ABTS^{\bullet+}$) were obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{\bullet+}$ was diluted in ultrapure water until absorbance at 734 nm was 0.7 (±0.1). HT, TY and Trolox™ (TR) (as antioxidant reference) was dissolved in ethanol to yield a 10 mM stock solution and diluted with ultrapure water to the assayed concentrations. Twenty microliters of each concentration of HT, TY, standard (TR), blank (ultrapure water) or ethanol control (8%) were added to a 96-well plate. The reaction was initiated by the addition of 280 µL of $ABTS^{\bullet+}$. Absorbance readings were taken every 5 min at 30 °C for 2 h in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate.

The inhibition of $ABTS^{\bullet+}$ was calculated according to the percentage of Radical Scavenging Activity (% RSA) described above (at t = 30 min).

2.4. ORAC Assay

Peroxyl radical scavenging activity was measured by the $ORAC_{FL}$ assay as previously described [14]. A stock solution of HT or TY were reconstituted in DMSO and then diluted in PBS. A stock solution of TR, as antioxidant standard, was also diluted in DMSO and diluted in PBS. The assay was carried out in 96-well plates with a final volume of 160 µL. Samples were run in triplicate. Fluorescein (48 nM) was mixed with various concentrations of SQ, standard (TR) or blank (PBS) containing at final volume 1% (v/v) DMSO. Plates were incubated for 15 min at 37 °C. The assay was initiated by the addition of AAPH (100 mM) and fluorescence readings (Ex: λ_{485}/ Em: λ_{520} nm) were taken every
5 min at 37 °C for 160 min in a microplate reader (TECAN GENios Plus). Final results were calculated based on the difference in the Area Under the fluorescence decay Curve (AUC) between the blank and each sample. The AUC formula was:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{20}/f_0$$

Results were expressed as micromolar TR equivalents (TE) calculated using the line equation from the standard curve:

$$TE = (Y - b)/m$$

where Y is the net AUC ($AUC_{sample} - AUC_{control}$), m is the slope and b is the Y-intercept.

2.5. Cell Culture

Highly invasive MDA-MB-231 human breast cancer cells (estrogen and progesterone receptor-negative), minimally invasive MCF7 human breast cancer cells (estrogen and progesterone receptor-positive) and immortalized non-tumorigenic MCF10A human breast epithelial cells, were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Breast tumor cells were grown as a monolayer culture in Minimum Essential Medium with Eagle's salts (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Hepes Buffer 1 M, 1% Sodium Pyruvate 100 mM and 1% Non-Essential Amino Acids mixture 100×. MCF10A cells were cultivated in HuMEC Ready Medium. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO_2. Cells were routinely sub-cultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments.

2.6. Cell Proliferation Assay

Cell proliferation, measured as the cellular growth of treated cells *vs.* untreated controls, was measured using an XTT-based assay as described by Scudiero *et al.* [15] with some modifications. Briefly, cells were seeded at 2 × 10^3 cells/well (MCF7) or 1 × 10^3 cells/well (MDA-MB-231 and MCF10A) into 96-well culture plates (flat bottom) (100 µL of cell suspension/well). At 24 h after plating, 100 µL of fresh culture medium, with different concentrations of HT or TY was added in triplicate to the wells. Plates were incubated for 24 h or 24 h followed by a 48 h proliferation period with fresh medium at 37 °C and 5% CO_2. At these time points, medium was removed and 200 µL of fresh RPMI medium without phenol red that contained XTT (200 µg/mL) and PMS (20 µg/mL) was added. Plates were incubated for 3

h at 37 °C in 5% CO_2 and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

$$\text{viable cells (\%)} = (OD_{\text{treated cells}}/OD_{\text{control}}) \times 100$$

where OD is the difference in absorbance between optical density units ($OD = OD_{450} - OD_{620}$).

All measurements were performed in triplicate and each experiment was repeated at least three times.

2.7. Cell Cycle Assay

Cells were seeded in 12-well culture plates at 1×10^5 cells/well for MCF7 and MDA-MB-231 or at 5×10^4 cells/well for MCF10A for 48 h. Cells were then treated with different doses of HT or TY for 24 h. After incubation, cells were washed in cold PBS, fixed with cold 70% ethanol and stored at −20 °C for at least 24 h. At least 1×10^4 cells per sample were analyzed on an EPICS XL-MCL (Beckman Coulter, Spain) flow cytometer after propidium iodide labeling (PI/RNase Staining Buffer kit). The percentage of cells in G_0/G_1, S and G_2/M phases were calculated using FlowJo program (v5.7.2). Each experiment was repeated three independent times.

2.8. Apoptosis

The percentage of apoptosis was determined using a double staining assay with FITC-conjugated Annexin V and propidium iodide (PI). Briefly, after 24 h of cell exposure to the previously indicated doses of HT or TY in 12-well culture plates, cells were harvested, washed twice in cold PBS and resuspended in 100 µL 1× Annexin Binding Buffer. Cells were then stained with 5 µL Annexin V-FITC and 1 µL PI solution, gently vortexed, and incubated for 15 min at room temperature in the dark before flow cytometric analysis. As a positive control, cells

were treated with 1 μM camptothecin (CPT). Each experiment was repeated three independent times.

2.9. Reactive Oxygen Species Detection

Intracellular reactive oxygen species (ROS) level was measured using a cell-permeable fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as we described previously [16]. In brief, cells were seeded into 96-well culture plates at 1×10^4 cells/well (MCF7, MDA-MB-231 cells) or 5.5×10^3 cells/well (MCF-10A cells). After 24 h at 37 °C and 5% CO_2, cells were treated with different doses of HT or TY for 24 h. Cells were then washed twice with Hank's Buffered Salt Solution (HBSS) and incubated with fresh DCFH-DA (100 μM) in HBSS for 30 min at 37 °C in 5% CO_2. DCFH-DA stock solution (20.5 mM) was prepared in DMSO and stored at −20 °C for maximum one month. After that, cells were washed twice in HBSS, and wells were filled with 100 μL HBSS before fluorescence acquisition in a plate reader (TECAN GENios Plus) (Ex: λ_{485}/Em: λ_{535} nm, Gain 60). Intracellular ROS level percentage was calculated as follows:

$$F = [(F_{t_{30}} - F_{t_0})/F_{t_0}] \times 100$$

where F_{t_0} is the fluorescence at t = 0 min and $F_{t_{30}}$ the fluorescence at t = 30 min.

It has been reported that the addition of H_2O_2 increases oxidative stress in cultured cells [17]. Therefore, in order to evaluate the protective capacity of HT or TY against induced oxidative stress, H_2O_2 (500 μM) was added to the wells after removal of assay medium. This allows avoiding a direct reaction in the medium between these compounds and the oxidant source. After 30 min at 37 °C, fluorescence was quantified as described above.

All tests were run in triplicate for each experimental condition and each experiment was repeated at least three times. All experiments were conducted using iron-free media (MEM and HuMEC).

2.10. Alkaline Single-Cell Gel Electrophoresis (Comet Assay)

At 24 h, cells treated with HT or TY were scraped into 12-well culture plates, washed twice (300× *g* 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free) and resuspended in 1 mL of cold 1× PBS. In order to evaluate the ability of HT and TY to prevent oxidative DNA damage, cell suspensions were exposed for 10 min to 50 µM H_2O_2 at 4 °C. After that, cells were washed twice and frozen in FBS-DMSO (90:10, v/v) at −80 °C until the Comet assay procedure.

DNA single strand break by alkaline microgel electrophoresis was performed according to Singh et al. [18] with some modifications. Cells were thawed in a bath at 37 °C, centrifuged (300× *g* 10 min, 4 °C) in cold MEM with 25% FBS and resuspended in cold 1× PBS to a density of 1.65 × 10^5 cells/mL. Cells were then suspended in melted and cooled (at 40 °C) low melting point agarose (LMA). Cell suspensions (50 µL) were spread over a sample area of pre-warmed 1% normal melting point agarose (NMA) precoated CometSlide™ slides. After 15 min at 4 °C in the dark, slides were immersed in cold Lysis Solution (Trevigen, Inc.) at 4 °C for 30 min to dissolve lipids and proteins. In order to separate the two DNA strands, slides were then immersed in fresh Alkaline Solution (pH > 13) for 30 min at room temperature in the dark. Electrophoresis was performed in an Ebony acrylic electrophoresis tank with a cooled platform containing cold Alkaline Electrophoresis Solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 25V (1 V/cm) and 300 mA for 40 min. The slides were washed twice with distilled water for 10 min and neutralized with 10 mM Tris-HCl, pH 7.5 for 5 min, followed by immersion in 70% ethanol for 5 min and air-dried overnight at room temperature. Slides were stained with Sybr® green before scoring.

2.11. Slide Scoring and Analysis

DNA strand breaks were examined using a fluorescence microscope (Zeiss Axiovert 200) equipped with a Luca EMCCD camera (Andor Technology, Belfast, UK) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). Fifty cell images were randomly characterized per sample using 20× magnification. Relative fluorescence between head and tail through the olive tail moment

(Olive_TM) was used to determine DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

$$\text{Olive\_TM} = [(\text{Tail (mean)} - \text{Head (mean)}) \times \text{Tail (\% DNA)}]/100$$

2.12. Statistical Analysis

Results are presented as mean (±SEM), except for cell proliferation results. For this assay, results are presented as mean (±SD). Results are expressed as a percentage relative to the control, which was defined as 100%. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Values of $p < 0.05$ were considered significant. Statgraphics Plus 5.1 statistical software (Statpoint Technologies, Inc., Warrenton, VA, USA) was used for the statistical analysis.

3. Results

3.1. Effect of HT and TY on Radical Scavenging Activity

Radical scavenging capacity was determined using DPPH, ABTS and ORAC assays.

The antiradical activity of HT and TY, measured by scavenging activity in the DPPH radical assay, indicated that HT at up to 10 mole ratio (mole antioxidant/mole DPPH) exerts a slightly higher scavenging activity than TOC while TY does not possess a radical scavenger activity (Table 1(a)).

The ABTS antiradical assay showed that HT was more effective than TR in scavenging the ABTS cationic radical while TY exhibited a maximum 85% RSA at 800 μM (Table 1(b)).

The peroxyl radical scavenging activity of HT and TY, measured by the $ORAC_{FL}$ assay, showed a protective effect against AAPH-induced peroxyl radical activity for both phenols. Both exerted higher protection against the peroxyl radical than TR for low concentrations up to 100 μM (Table 1(c)).

3.2. Cell Proliferation

To investigate the effect of HT and TY on human breast cell growth, cells were treated with concentrations of HT or TY ranging from 1 to 100 μM for 24 h. Neither HT nor TY had significant effects on the cell proliferation rates of MCF7, MDA-MB-231 and MCF10A cells (Figure 1(a)), even after an additional 48 h with fresh medium (Figure 1(b)). We also investigated the potential antiproliferative effect of these compounds at high, non-physiological concentrations up to 1000 or 5000 μM of HT or TY, respectively. HT showed a dose-dependent reduction of cell proliferation in the three cell lines from a concentration of 200 μM with an absence of viability observed at 1000 μM, while TY did not affect cell viability at any concentration assayed (data not shown).

No marked changes in cell morphology were observed by light microscopy in any of the cell lines tested when concentrations between 1 and 100 μM of HT or TY were used (data not shown).

3.3. Cell Cycle and Apoptosis

To evaluate whether HT or TY interfered with the cell cycle or the induction of apoptosis, MCF7, MDA-MB-231 and MCF10A cells were treated for 24 h with increasing concentrations of HT or TY (between 10 and 200 μM). The results revealed that HT and TY did not alter the cell cycle in any of the cell lines studied (data not shown).

Flow cytometric analysis of apoptosis revealed that treatment with HT or TY for 24 h did not induce apoptosis in MCF10A cells or in MCF7 or MDA-MB-231 cells when compared to the controls (data not shown).

3.4. Intracellular ROS Level

Intracellular reactive oxygen species (ROS) were quantified by the dichlorofluorescein diacetate (DCFH-DA) assay using a microplate reader. Results showed a dose-dependent decrease in ROS level of MCF10A cells treated for 24 h with either HT or TY. However, HT and

TY failed to significantly decrease intracellular ROS level in either MCF7 or MDA-MB-231 cells (Figure 2(a)). While HT reduced ROS level by up to 20% in MCF7 cells, this reduction was not considered statistically significant (p = 0.34).

H_2O_2 effectively induced oxidative stress in both, human breast cancer cells and human breast epithelial cells (Figure 2(b)). In order to investigate the *in vitro* preventive effect of HT or TY against H_2O_2-mediated oxidative stress, we measured the intracellular ROS level in cells treated with HT or TY for 24 h. As can be seen in Figure 2(c), MCF10A cells treated with HT or TY showed a significant dose-dependent decrease in ROS production compared to the control. In addition, HT was also able to decrease the ROS level in MCF7 and MDA-MB-231 cells induced by H_2O_2 exposure. It is worth mentioning that the decrease in ROS level was greater in the breast epithelial cell line than in the breast cancer cell lines. On the other hand, TY did not decrease ROS level in MCF7 or MDA-MB-231 cells at concentrations up to 5000 μM (Figure 2(c)).

IC_{20} and IC_{50} values were defined as the values for 20% and 50% antioxidant inhibition of basal or H_2O_2-stimulated fluorescence in DCFH-DA probes. The Relative Antioxidant Value (RAV) ratio was found to be a good parameter for the determination of oxidative inhibition profiles.

$$RAV = [(IC_{20\,(PH)}/IC_{20\,(TOC)}) + (IC_{50\,(PH)}/IC_{50\,(TOC)})]/2$$

where PH is the compound (simple phenol) and TOC is the reference (α-tocopherol).

Our results showed that TY has a RAV about 46-fold higher than TOC for MCF10A cells whereas HT only has 1.44-fold higher. This indicated much high antioxidant activity of HT compared with TY in normal breast cells, but less than TOC (Table 2). In MCF7 and MDA-MB-231 cells, a 50% antioxidant inhibition was not observed; therefore, RAV ratios were not determined in these cell lines.

Interestingly, in H_2O_2-stimulated MCF10A cells, the RAV ratio of TY was 42-fold higher than TOC, indicating a very low antioxidant capacity in normal breast cells. The 0.67-fold difference between RAV ratio of HT and TOC is of particular interest, due to the high antioxidant activity of HT in H_2O_2-stimulated MCF10A cells (Table 2).

3.5. Effect of HT and TY on Oxidative DNA Damage

The ability of H_2O_2 to induce DNA strand breaks in these human breast epithelial cell lines was examined using the Comet assay. In untreated cells, DNA does not migrate far from the origin when examined by alkaline microgel electrophoresis (Figure 3(a)). Following H_2O_2 exposure, control and pretreated breast cells with damaged DNA have the shape of a comet, the tail length and fluorescent intensity of which are related to the number of DNA strand breaks induced by the DNA-damaging agent (Figure 3(b,c)).

Breast cells exposed to H_2O_2 were effectively DNA damaged and the mean olive tail moment (Olive_TM) was determined by the Comet assay. Breast epithelial cells were the most sensitive to the H_2O_2-induced DNA damage (Figure 4(a)).

In unexposed cells, HT reduced DNA damage significantly in MCF7, MDA-MB-231 and MCF10A cells whereas TY only reduced it in MCF10A cells (Figure 4(b)). In H_2O_2-exposed cells, HT showed a preventive DNA damage effect in the three cell lines whereas TY was unable to reduce Olive_TM in any of the cell lines; indeed, in MDA-MB-231 cells, TY increased Olive_TM significantly.

4. Discussion

There is some scientific evidence relating Mediterranean dietary pattern with a lower incidence of cardiovascular diseases and cancer, among other diseases. Virgin olive oils (VOOs) represent the main source of fats in this diet and it has been demonstrated that consumption of VOOs reduces human arterial hypertension, lipid peroxidation of membranes, tumor incidence and number of tumors [19,20]. Minor compounds play a key role in VOOs' healthy properties. Among them, phenols have demonstrated healthy bioactivity properties. Interest in phenolic compounds has increased greatly, with attention being focused on finding naturally occurring antioxidants for foods or medical uses to replace synthetic antioxidants that, in some cases, have been reported to be carcinogenic [21].

HT and TY are two of the major simple phenols present in VOOs as simple form or conjugates [2]. Bioavailability studies have demonstrated that they are dose-dependently

absorbed in animals and humans after olive oil ingestion [22], accumulated in the body and, finally, systemically exert biological effects [23].

The present work describes the antioxidant capacity of HT and TY molecules using chemical and cellular assays and their relationship with proliferation of human breast tumor *vs.* normal cells.

HT and TY are structurally identical except that HT has an extra –OH group forming a catechol group, which is considered responsible for its higher antioxidant activity. This catechol group is able to stabilize free radicals through the formation of intermolecular hydrogen bonds [8]. In our chemical analysis, the catechol phenol HT exhibited a strong antioxidant activity in DPPH, ABTS and ORAC assays, while TY, without a catechol group, showed a weak antioxidant activity in DPPH assay. Remarkably, TY acts as an efficient scavenger against ABTS and AAPH radicals, although to a lesser extent than HT, indicating the minor importance of the cathecol group in cationic or peroxylic radicals' scavenging activities. These results are in agreement with those previously reported by Visioli et al. [8] affirming that HT and, to a lesser degree, TY are more potent scavengers of free radicals than vitamin E.

Although nowadays there is no scientific evidence relating to the physiological concentrations of HT or TY after olive oil ingestion, some authors have suggested it could be between 10 and 100 μM [24]. Cell treatment with HT or TY in the range of their possible physiological concentrations (1–100 μM) did not have any effect on cell proliferation in any of the cell lines studied, independently of the exposure times. However, HT dramatically reduced the viability of MCF7, MDA-MB-231 and MCF10A cell lines when used at concentrations from 200 μM to 1000 μM. Fabiani et al. described such an effect in colon adenocarcinoma HT29 cells [25]. Furthermore, HT and TY did not alter the cell cycle or induce apoptosis in these cell lines. Although these results are in agreement with those achieved in LLC-PK1 renal cells, they are in contrast with results in human promyelocytic leukaemia HL60 cells with a noticeable antiproliferative, cell cycle arrest and apoptotic effect of HT. Otherwise, TY showed no antiproliferative effect in HL60 cells [7,25].

HT or TY's inability to inhibit breast cancer cell proliferation at the assayed times and concentrations, suggests that they cannot protect against breast cancer once developed. Quiles et al. [24] described the lack of inhibition of HT or TY in PC3 cells treated with 10 to 250 μM, as did Menendez et al. [26] in SKBR3 and MCF7 cells after 5 days of HT or TY treatments in the range of 6.25 to 100 μM. Moreover, Sirianni et al. [27] recently described the dose-dependent

inhibition of MCF7 cell proliferation by HT and oleuropein (OL) with treatments of 1 to 100 μM; cell growth was induced by 17-β-estradiol (E_2). In addition, HT and OL are not able to interfere with estrogen action through competition with estrogen receptors (ER), which are responsible for activation of the gene expression involved in cell proliferation.

In order to clarify how nutritional antioxidants are able to prevent or treat oxidative damage, Berger [28] affirmed that nutrients cannot treat an installed disease, such as gastrointestinal cancer, but that they may prevent its promotion. Indeed, the answer to the question: "Can installed damage caused by ROS be treated by antioxidant nutrients?" is "probably not", but the answer to the question: "Can oxidative damage be treated nutritionally?" is "yes" [28].

Growing evidence supports the hypothesis that risk factors such as lifestyle, age, environment, diet, drinking, smoke, *etc.* are determinants in breast neoplastic transformation, and are closely associated with a chronic increase in the basal level of oxidative stress. A decrease in oxidative stress state could prevent the development of tumors and, potentially, cancer. In fact, serum markers for oxidative DNA damage have been shown to increase in women diagnosed with breast cancer [29]. On the other hand, it has been suggested that consumption of VOOs, which are particularly rich in phenolic antioxidants, such as HT and TY, should afford considerable protection against breast cancer by inhibiting oxidative stress [2]. In our study we demonstrated that HT and TY reduce basal and H_2O_2-induced ROS level in breast epithelial MCF10A cells, whereas TY failed to reduce both in MCF7 or MDA-MB-231 cells and HT only reduced H_2O_2-induced ROS level slightly in breast cancer cells. These results point to a differential antioxidant activity of both compounds between normal breast and tumor cells as we described for squalene [16]. Thus, we suggest that HT and TY could prevent oxidative stress in normal breast cells, thereby preventing the initiation of a chain of reactions to transform normal cells into cancer cells. Noticeably, it is necessary to use a much larger amount of TY to obtain the same ROS reduction level as HT in MCF10A cells (Table 2). Up to 100 μM concentrations of HT and TY used in the present study are probably within the physiological range. However, 500 to 5000 μM of TY exceed this range and could be regarded as being in the pharmacological range.

Di Bendeto *et al.* [11] described differences between HT and TY in inhibiting cell-mediated oxidation of LDL (100% HT *vs.* 40% TY) in J774 A.1 macrophage cells due to its intracellular presence. Thus, time-dependent TY, accumulated inside the cell was effective only at later time-

points (24 h) or at higher concentrations than HT, which was rapidly found inside the cells and disappeared within 18 h. Thus, we can presume a quick antioxidant defense by HT followed by a slower defense by TY upon VOO intake.

Estrogens, known human breast pro-carcinogens, exert their actions by two mechanisms; the ER-dependent mechanism, involving the activation of ER and subsequent stimulation of cell growth and proliferation [30] or the ER-independent mechanism, involving the generation of genotoxic estrogen metabolites, which are highly reactive and damage DNA by the formation of free radicals and consequently ROS [30]. In accordance with Sirianni *et al.* [27], HT inhibition of E_2-induced MCF7 proliferation does not involve the ER-dependent mechanism but points to an inhibition of the E_2 signaling pathway. Felty *et al.* [31] identified mitochondria as a major source of E_2-induced ROS (mtROS) in breast cancer cells and described mtROS as a messenger involved in signaling pathways of cell proliferation control, increasing the transcription of cell cycle genes. These authors found the same amount of mtROS in ER-negative MDA-MB-468 cells and in ER-positive MCF7 or T47D cells, suggesting that mtROS production does not depend on the presence of ER in breast cancer cells. If mtROS acts as a messenger in breast cancer proliferation, it could explain why an antioxidant such as HT reduces E_2-induced cell proliferation, as described by Sirianni , whereas in the same concentrations without E_2 stimulation we do not detect any significant growth alteration.

Cellular protection against oxidative stress is provided by two types of antioxidants; direct antioxidants with a redox activity; and indirect antioxidants (redox active or not) which activated the Nrf2/ARE pathway resulting in transcription of phase II enzymes such as glutathione S-transferase, NAD(P)H: quinone oxidoreductase 1 or glutathione reductase [32]. In addition to the fact that HT and TY act as direct antioxidants, they could also be indirect antioxidants activating the nuclear factor-like 2 (Nrf2). Nrf2, considered a key factor in the cellular defense mechanisms against oxidative stress, might be induced more strongly in MCF10A cells than in MCF7 and might have little or no effect in MDA-MB-231 cells, explaining the differential protection effect of HT and TY on intracellular ROS level. To the best of our knowledge, until now, only Liu *et al*. [33] have described the protection of HT on ARPE-19 human retinal pigment epithelial cell line from oxidative stress induced by acrolein, a major component of cigarette smoke. Further studies will be necessary to elucidate the possible Nrf2/ ARE pathway intervention of HT and TY differential antioxidant activity in breast cell lines.

HT has been described as preventing DNA damage beyond its antioxidant capacity, as it can affect a range of enzymes, including cyclooxygenase and NAD(P)H oxidase while TY has no protective effect [8]. In accordance with these authors, our findings point to a protective effect of HT against basal and H_2O_2-induced DNA damage regardless of the breast cellular type, whereas TY only has a protective effect on ROS basal level in non-tumoral breast cells.

Both compounds reduce intracellular ROS level and oxidative DNA damage in normal breast cells. This could protect against cellular mutations, preventing carcinogenesis. However, when the disease has occurred, oxidative status in the malignancy place is altered. In this condition, while HT still protects non-tumor breast cells against DNA damage, TY fails to protect them at physiological concentrations. Although HT contributes to reduce DNA damage in normal breast cells, it protects breast tumor cells too. Accordingly, our results must be interpreted carefully, because a reduction of DNA damage in cancer cells might promote cell growth and might inhibit the action of anthracycline chemotherapeutic agents, such as doxorubicin, which induces apoptosis of cancer cells by the oxidative damage resulting from enhanced oxidative state of the cells or, in contrast, might reduce ROS messenger signaling of proliferation resulting in a reduction of tumor cell growth. In any case, we have not detected any modulation of the growth activity *in vitro* in breast cancer cells after HT or TY treatment at the assayed times.

In this paper, HT has been described as an antioxidant compound with higher activity than TY and related to the prevention of breast cancer, but we must not forget that VOO´s minor compounds can interact with each other, potentiating or inhibiting the effects described for each component alone. Our results indicate some healthy properties of these two simple phenols which may be of interest in pharmacology or as a nutritional supplement or could even lead to establishing the ideal concentrations of each component in VOOs in order to label it as a healthy oil. However, we must be prudent about extrapolating these results regarding epidemiological olive oil health impacts. Future work is needed to investigate these synergetic or inhibitory effects.

5. Conclusions

The simple phenol HT could contribute to the preventive cancer activity attributed to VOOs due to the reduction of oxidative stress and oxidative DNA protection in normal breast cells at

physiological concentrations, whereas TY is needed at pharmacological concentrations to reduce oxidative stress and fails to protect DNA damage against an oxidative burst.

Both phenols exert a selective antioxidant defense, preventing oxidation in normal breast cells but not in breast cancer cells, which could be helpful to cancer therapies that increase oxidative stress. HT also prevents induced DNA damage in cancer cells, so it might interfere with these therapies.

Although *in vitro* studies have pointed to a preventive role of HT against human breast cancer, the precise mechanisms of action remain to be clarified. Further studies are necessary to elucidate the cellular signaling events that HT and TY target in oxidative stress protection and subsequent breast cancer prevention.

Acknowledgments

This study was supported by the "Instituto Andaluz de Biotecnología" (BIOÁNDALUS 08/22/ L5.3); Ministerio de Ciencia e Innovación-FEDER (RTA2008-00066-C03-03); and "Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud" (CEAS).

Conflict of Interest

The authors declare no conflict of interest.

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Table 1 (a)

mole AH/ mole DPPH	HT	TY	TOC
0	3.96	3.64	3.63
0.06	36.24	n.d.	23.21
0.13	71.47	5.20	48.16
0.25	96.40	4.25	75.85
0.5	97.80	3.02	90.17
1	98.07	2.59	95.98
2.5	n.d.	2.34	n.d.
5	n.d.	3.44	n.d.
10	n.d.	3.37	n.d.

n.d.: not determined.

Table 1 (b)

µM	HT	TY	TR
6	5.07	10.31	n.d.
12.5	5.45	14.32	n.d.
25	8.42	20.14	n.d.
50	18.09	31.81	16.21
100	36.63	44.55	29.39
200	69.53	58.25	50.07
400	96.30	73.82	88.03
800	99.47	85.04	99.48

n.d.: not determined.

Table 1 (c)

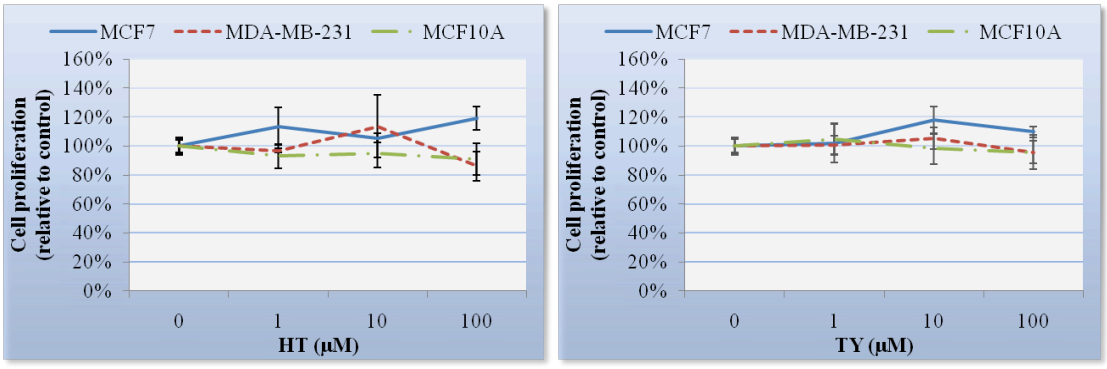
TE (µM)	HT (µM)	TY (µM)
3.12	14.82	1.64
6.25	30.97	8.30
12.5	50.37	20.84
25	92.24	58.35
50	150.43	106.76
100	262.76	205.57

Table 2

	Basal			H_2O_2-stimulated		
	HT (µM)	TY (µM)	TOC (µM)	HT (µM)	TY (µM)	TOC (µM)
IC_{20}	3.52	4.04	4.33	2.66	65.36	2.49
IC_{50}	65.64	2942.60	31.89	20.66	4244.40	73.50
RAV	1.44	46.60	1.00	0.67	42.00	1.00

Figure 1

(a



(b

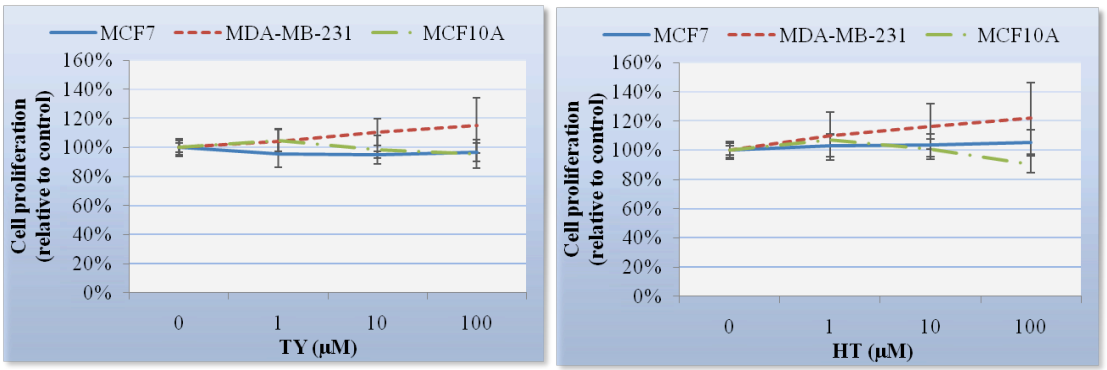
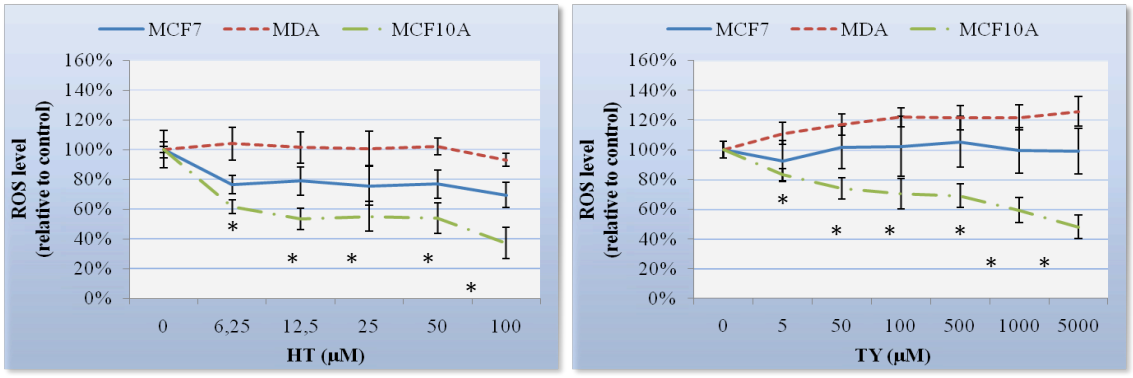
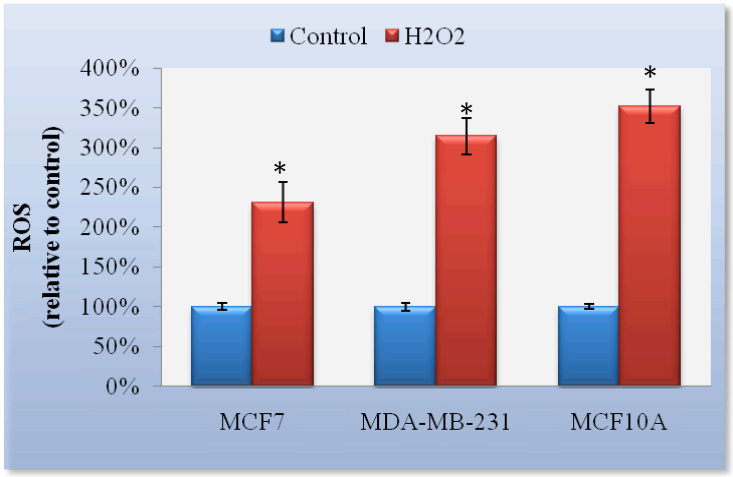


Figure 2

(a


MCF7
MDA
MCF10A
ROS level (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
6,25
12,5
25
50
100
HT (µM)
MCF7
MDA
MCF10A
ROS level (relative to control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
5
50
100
500
1000
5000
TY (µM)

(b


Control
H2O2
ROS (relative to control)
400%
350%
300%
250%
200%
150%
100%
50%
0%
MCF7
MDA-MB-231
MCF10A

(c

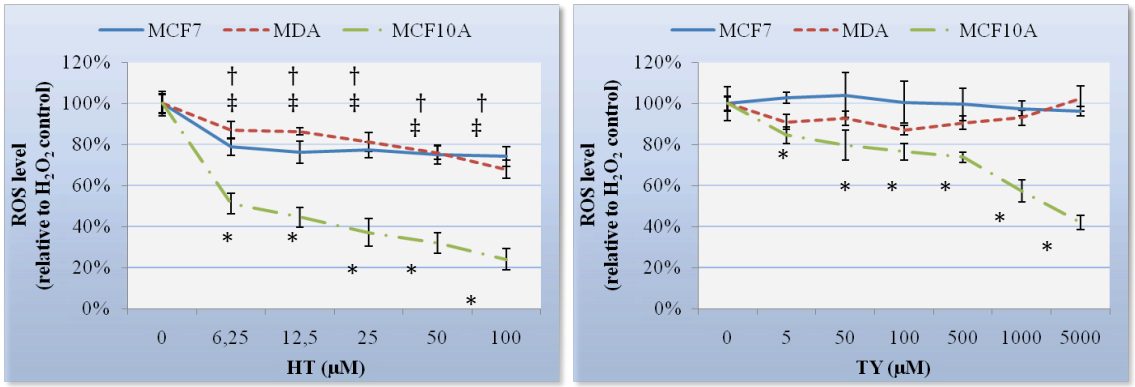

MCF7
MDA
MCF10A
ROS level (relative to H2O2 control)
120%
100%
80%
60%
40%
20%
0%
0
6,25
12,5
25
50
100
HT (µM)
MCF7
MDA
MCF10A
ROS level (relative to H2O2 control)
120%
100%
80%
60%
40%
20%
0%
0
5
50
100
500
1000
5000
TY (µM)

Figure 3

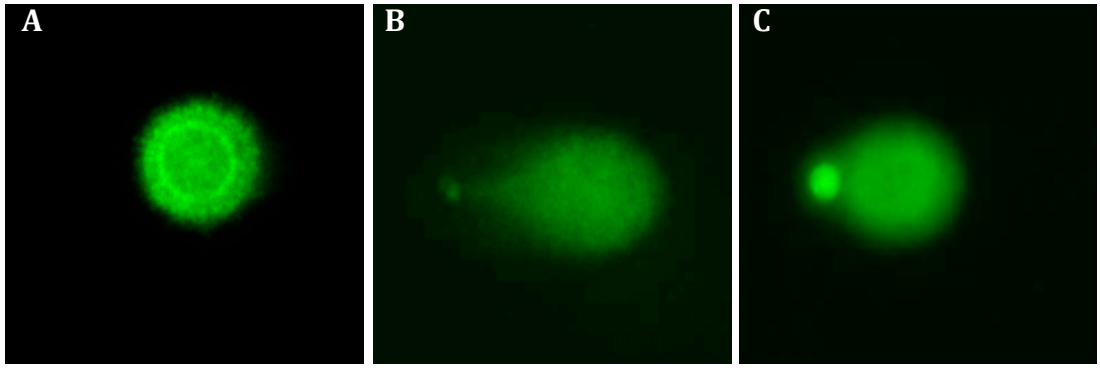
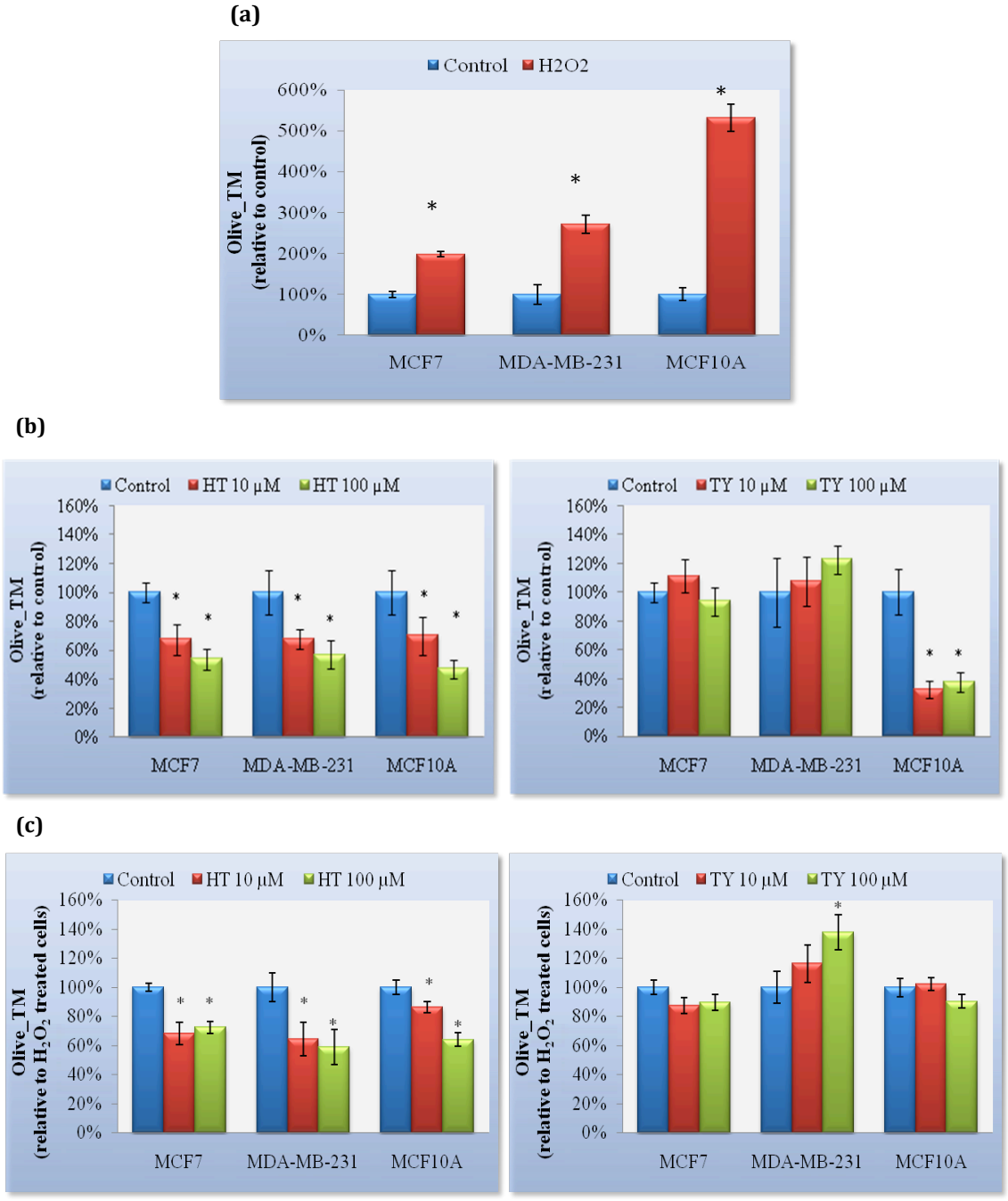


Figure 4



Legends:

Table 1. Antioxidant activity of hydroxytyrosol (HT) or tyrosol (TY) quantified as Radical Scavenging Activity (RSA) by (**a**) DPPH assay (% RSA at 50 min) and (**b**) ABTS assay (% RSA at 30 min); (**c**) Antioxidant activity quantified as Trolox Equivalent (TE) by $ORAC_{FL}$ assay. Trolox™ (TR) and α-tocopherol (TOC) were used as antioxidant references.

Table 2. Oxidative inhibition in MCF10A cells. IC_{20} and IC_{50} values defined as the values for antioxidant inhibition of basal or H_2O_2-stimulated fluorescence in DCFH-DA assays and the Relative Antioxidant Value (RAV) as a parameter for the relative determination of oxidative inhibition profiles compared to α-tocopherol.

Figure 1. Cell proliferation assay measured with XTT tetrazolium salt (**a**) after 24 h of HT or TY exposure, or (**b**) after 24 h of HT or TY exposure followed by 48 h with fresh medium. Data are the mean (±SD) relative to an untreated control of three independent assays carried out in triplicate.

Figure 2. (**a**) Intracellular reactive oxygen species (ROS) in breast cells treated for 24 h with HT or TY; (**b**) Increase of the cellular ROS level after an oxidative burn with H_2O_2; (**c**) Intracellular ROS in breast cells treated for 24 h with HT or TY followed by an oxidative burst with H_2O_2. Inhibitory effects of HT and TY are shown as percent inhibition of untreated or H_2O_2-stimulated fluorescence and represented as the mean ± SEM of three independent replicates carried out in triplicate. † MCF7; ‡ MDA-MB-231; * MCF10A indicates significant differences.

Figure 3. Representative images of Comet assay analysis of MCF10A cells. (**a**) Untreated cell, showing a circular shape indicating absence of DNA damage; (**b**) 10 min. H_2O_2 exposed cell, exhibiting a long and bright tail related to DNA strand breaks, indicating DNA oxidative damage; (**c**) 10 min. H_2O_2 exposed cell after 24 h of 100 μM HT pretreatment, illustrating the reduction of tail length and fluorescent intensity indicative of reduced DNA damage.

Figure 4. Olive Tail Moment (Olive_TM) as the mean ± SEM for three independent assays. (**a**) After an H_2O_2 injury; (**b**) after 24 h of HT or TY treatment, and (**c**) after 24 h of HT or TY treatment followed by an H_2O_2 injury.

RESUMEN GLOBAL DE RESULTADOS.

RESUMEN GLOBAL DE RESULTADOS.

1. Artículo: Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells.

1.1. Actividad antioxidante del escualeno

Para conocer en profundidad la actividad antioxidante de esta molécula se realizaron una serie de ensayos químicos antioxidantes basados tanto en la transferencia de electrones (DPPH y ABTS) como en la transferencia de átomos de hidrogeno (ORAC) en un rango amplio de concentraciones que nos permitieran caracterizar las propiedades antioxidantes de esta molécula.

Se evaluó su capacidad de reducir al radical libre DPPH, a ratios molares 10:1 (escualeno:DPPH) con un RSA (radical scarvenging activity) inferior a un 5 %, indicando su total ausencia de actividad comparado con el RSA del Trolox de un 50 % a un ratio molar 0,16:1 (Trolox:DPPH).

El radical catiónico $ABTS^{\cdot+}$ nos muestra la capacidad de la molécula antioxidante ensayada para reducir radicales catiónicos comparado con el Trolox. En estas pruebas, el RSA del escualeno, ensayado hasta 800 µM, no superó el 4% comparado con el 50% de RSA del Trolox a 143,57 µM.

La actividad antioxidante del escualeno ensayada como capacidad de reducir radicales peroxilo, nos mostró igualmente su total ausencia de actividad ensayada hasta 400 µM y comparada con la del Trolox. Al ser su actividad prácticamente 0, no pudimos expresarla como equivalentes de Trolox (TE) como es habitual en estos ensayos.

El estudio de la capacidad antioxidante nos ha mostrado que el escualeno no posee actividad antioxidante *per se* en ninguno de los ensayos realizados.

1.2. Modulación de la proliferación celular del escualeno sobre células humanas tumorales de mama MCF-7 y MDA-MB-231 y no tumorales MCF10A

Los estudios realizados sobre el efecto del escualeno en la proliferación celular tras 24, 48 o 120 h de exposición a concentraciones entre 3,12 y 50 μM seguidos de un período proliferativo han dado como resultado la ausencia de variación en el crecimiento celular de las lineas celulares MCF-7 y MCF-10A en cualquiera de los tiempos de exposición y a cualquier concentración ensayada. En el caso de la línea MDA-MB-231 incluso ha habido un ligero incremento del crecimiento celular aunque no es significativo. Estos resultados nos han demostrado como el escualeno no parece alterar significativamente el crecimiento celular de las líneas tumorales ni de la no tumoral ensayadas.

A pesar de no haber encontrado alteraciones en la proliferación celular, se estudió el efecto del escualeno sobre el ciclo celular y sobre la inducción de apoptosis por citometría de flujo con concentraciones hasta 4 veces superiores a las ensayadas en la proliferación celular para cerciorarnos de que el escualeno no alteraba de forma subyacente a estas células. Los resultados confirmaron los resultados obtenidos en los ensayos de proliferación celular con una ausencia total de modulación del ciclo celular normal de las líneas tumorales MCF-7 y MDA-MB-231 y de la no tumoral MCF10A, así como ningún indicio de inducción de la apoptosis ni del incremento de necrosis celular en ninguna de las líneas celulares ensayadas.

1.3. Acción diferencial del escualeno sobre el estrés oxidativo en líneas tumorales y no tumorales de mama

El estrés oxidativo celular depende del equilibrio entre las EROs y los agentes antioxidantes endógenos y exógenos presentes en la célula. Como medida de ese equilibrio se pueden determinar los niveles intracelulares de EROs presentes en la célula, cuantificados como radicales libres del oxígeno mediante la transformación del DCFH-DA en DCF^{+}. Estos ensayos revelaron un importante efecto diferencial entre las líneas tumorales y la no tumoral. El escualeno, incapaz de reducir los niveles basales de EROs en las células tumorales, actuaban como antioxidantes intracelulares en la línea no tumoral MCF10A de manera dosis dependiente, logrando reducir en más de un 50 % las EROs basales a 50 μM.

Es conocido el efecto pernicioso que conlleva el estrés oxidativo crónico en pacientes que cursan con esta patología, y su relación con la inducción del cáncer, por lo que para conocer si esta acción diferencial se manifestaba igualmente en un estado oxidativo elevado, este fue inducido por el H_2O_2 como agente generador de EROs intracelular. Los resultados evidenciaron que el escualeno actuaba como un potente agente antioxidante en la línea no tumoral mientras que no modificaba los elevados niveles de EROs de las dos líneas tumorales.

He de señalar que en todos estos ensayos se utilizó la vitamina E, un potente y conocido antioxidante natural, como control. La vitamina E actuó como antioxidante tanto en los niveles basales como con los inducidos indistintamente sobre las tres líneas celulares, no presentando la acción diferencial descrita para el escualeno, que, sin ser *per se* una sustancia antioxidante, fue incluso mayor a la de la vitamina E tras la inducción oxidativa.

Las EROs intracelulares atacan y desestabilizan las moléculas con las que se encuentran por lo que pueden alcanzar el núcleo y producir daños en el ADN. Este efecto, si es leve puede ser corregido, pero cuando es mayor puede producir que la célula entre en apoptosis, o lo que es peor, que se transforme en una célula tumoral. Para determinar la potencial capacidad protectora del daño al ADN del escualeno frente al daño oxidativo, se recurrió a la electroforesis unicelular alcalina, mas conocida como “Comet Assay” por la forma de cometa resultante de las células dañadas. Tras incubar las células con diferentes concentraciones de escualeno durante 24 h, se expusieron a una inducción oxidativa con H_2O_2. Sobre la línea tumoral MCF-7 el escualeno no mostró ninguna acción, mientras que sobre la MDA-MB-231 produjo incluso un incremento del daño oxidativo al ADN (aunque no significativo). Nuevamente, el escualeno ejerció una acción diferencial protegiendo de manera dosis dependiente del daño oxidativo generado al ADN por el H_2O_2.

2. Artículo: Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells.

2.1. Actividad antioxidante del hidroxitirosol y del tirosol

Para determinar las propiedades antioxidantes del hidroxitirosol y del tirosol, se realizaron los ensayos químicos antioxidantes basados tanto en la transferencia de electrones (DPPH y

$ABTS^{\cdot+}$) como en la transferencia de átomos de hidrogeno (ORAC) en un rango amplio de concentraciones.

Los ensayos mostraron que el hidroxitirosol se comporta como un potente antioxidante al actuar como reductor del radical DPPH, del radical catiónico $ABTS^{\cdot+}$ y como protector de la actividad del radical peróxido inducido par AAPH. LA actividad del hidroxitirosol fue superior en los tres casos a la de sus controles antioxidantes (vitamina E y Trolox).

Bajo las mismas condiciones de ensayo, el tirosol no ejerció ninguna actividad sobre el radical DPPH, pero actuó como un potente reducto del radical catiónico ABTS y como protector de radicales peroxilos sobretodo a elevadas concentraciones.

Estos resultados nos muestran como estos dos componentes del aceite de oliva virgen son dos importantes antioxidantes naturales con un patrón de actuación singular, capaces de neutralizar los efectos de un desequilibrio oxidativo.

2.2. Modulación de la proliferación celular del hidroxitirosol y tirosol sobre células humanas tumorales de mama MCF-7 y MDA-MB-231 y no tumorales MCF10A

Para conocer los efectos del hidroxitirosol y del tirosol sobre la toxicidad celular, tratamos a las células con concentraciones entre 1 y 100 μM durante 24 h y para conocer su efecto sobre la proliferación se dejaron durante 48 h más con medio fresco.

En ninguna de las líneas celulares, tanto en las tumorales MCF-7 y MDA.MB.231, como en la no tumoral MCF10A, el hidroxitirosol o el tirosol han mostrado efecto alguno sobre el crecimiento celular. Solamente se han conseguido efectos antiproliferativos al tratar las células a concentraciones no fisiológicas de hasta 5000 μM. Se obtuvo una disminución de la proliferación dependiente de dosis del hidroxitirosol desde los 200 μM hasta los 1000 μM, concentración a la cual la supervivencia fue del 0%. Sin embargo, el tirosol no afectó al crecimiento celular ni siquiera a concentraciones tan elevadas.

A pesar de no haber encontrado alteraciones en la proliferación celular a dosis hasta 200 μM, se estudió el efecto del hidroxitirosol y del tirosol sobre el ciclo celular y sobre la inducción de apoptosis por citometría de flujo para cerciorarnos de que el hidroxitirosol y el tirosol no alteraban de forma subyacente a estas células. Estos ensayos mostraron una nula capacidad de modulación del ciclo celular de estos compuestos como la ausencia total de factores

apoptóticos o necróticos. La suma de estos resultados demuestran la ausencia de actividad del hidroxitirosol y del tirosol sobre el crecimiento celular de determinadas líneas humanas tumorales de mama y no tumorales.

2.3. Acción diferencial del hidroxitirosol y del tirosol sobre las EROs intracelulares en líneas tumorales y no tumorales de mama

La cuantificación de las especies reactivas del oxígeno intracelular con DCFH-DA reveló una disminución dependiente de dosis de los niveles basales en la línea no tumoral MCF10A tratada tanto con hidroxitirosol como con tirosol, efecto que no pudo encontrarse al realizarse estos ensayos sobre las líneas tumorales MCF-7 y MDA-MB-231.

Para determinar la acción preventiva de estos compuestos ante un choque oxidativo, las células pretratadas con los componentes del aceite fueron sometidas a una inducción oxidativa con H_2O_2. El hidroxitirosol fue capaz de paliar el incremento oxidativo en las tres líneas celulares de manera dependiente de dosis, aunque su acción fue mucho más acusada en la línea no tumoral MCF10A que en las tumorales MCF-7 y MDA-MB-231 con una disminución máxima próxima al 80% frente al 20% y 30% máximo alcanzado para MCF-7 y MDA-MB-231 respectivamente. El tirosol por su parte, no previno del incremento oxidativo a las células tumorales y sí a la no tumoral, aunque con un efecto mucho más moderado (reducción del 20% a 100 μM) aunque a concentraciones no fisiológicas sí alcanzó un efecto considerable sobre la línea MCF10A (disminución del 60% a 5000 μM).

Basándome en fórmulas aplicadas en otros campos de investigación para la comparación de los efectos de determinadas sustancias respecto a un control, determiné una fórmula para comparar los valores antioxidantes relativos basados en el IC_{20} e IC_{50} de los componentes a determinar comparados con los del de la sustancia de referencia. A este valor lo denominé Relative Antioxidante Value (RAV) y queda definido por la siguiente fórmula:

$$RAV= [(IC_{20\,(X)}/IC_{20\,(R)}) + (IC_{50\,(X)}/IC_{50\,(R)})]/2$$

donde IC_{20} es la concentración correspondiente a un 20% de reducción del nivel oxidativo, IC_{50} es la concentración correspondiente a un 50% de reducción del nivel oxidativo, X es la sustancia a determinar y R la sustancia de referencia.

De este modo podemos ver más fácilmente como en la línea no tumoral de mama MCF10A, el hidroxitirosol tiene un RAV de 1,44, comparado con el TOC (α-tocoferol), en las determinaciones basales y de 0,67 en las de inducción oxidativa indicando su alta capacidad antioxidante siendo superior incluso a la del TOC en este ultimo caso, mientras que el tirosol presenta un RAV de 46,6 y de 42 respectivamente señalando su capacidad más de 40 veces inferior a la del TOC para reducir o prevenir los niveles oxidativos intracelulares.

El daño oxidativo al ADN determinado por electroforesis unicelular alcalina, reveló que el hidroxitirosol actúa como un potente inhibidor del daño oxidativo al ADN tanto basal como inducido por H_2O_2 en las tres líneas celulares ensayadas, independientemente de su origen tumoral o no tumoral, mientras que el tirosol, con una actividad antioxidante intracelular en la MCF10A muy inferior a la del hidroxitirosol, mostró una elevada capacidad reductora del daño oxidativo basal al ADN desde la menor concentración ensayada (10 μM). Y a pesar de no prevenir del daño oxidativo al ADN inducido, sí aumentó un 40% el daño al ADN de las células tumorales altamente invasivas MDA-MB-231.

CONCLUSIONES Y PERSPECTIVAS

CONCLUSIONES

Las investigaciones realizadas para la elaboración de esta tesis doctoral nos han conducido a las siguientes conclusiones:

1. Se ha demostrado la ausencia de capacidad antitumoral de los fenoles simples hidroxitirosol y tirosol y del hidrocarburo escualeno frente a células tumorales de mama humanas.
2. Se ha demostrado la capacidad como agentes antioxidantes *per se* del hidroxitirosol y del tirosol y la ausencia total de actividad química antioxidante del escualeno.
3. Se ha demostrado que la actividad antioxidante intracelular del hidroxitirosol, tirosol y escualeno no depende directamente de las características químicas antioxidantes de las moléculas.
4. Queda demostrada la acción diferencial del hidroxitirosol, tirosol y escualeno relativa a sus actividad preventiva ante la oxidación y el daño oxidativo al ADN, al actuar sobre líneas de mama no tumorales y no sobre las tumorales de mama.
5. Nuestros resultados avalan que los tres compuestos minoritarios presentes en el aceite de oliva virgen estudiados; hidroxitirosol, tirosol y escualeno, a pesar de no mostrar actividad citotóxica o antiproliferativa sobre células tumorales, presentan capacidades preventivas frente al cancer de mama *in vitro* lo que sugiere su participación en los efectos beneficiosos del consumo de este aceite en la prevención del cancer de mama, ampliamente documentado y característico de países consumidores de una dieta mediterránea cuya fuente principal de grasas es el aceite de oliva virgen.

PERSPECTIVAS

Los resultados derivados de este proyecto nos han abierto la puerta de una nueva línea de investigaciones basada en el estudio comparativo entre modelos celulares tumorales y no tumorales, una metodología, que a pesar de no ser novedosa, no está muy extendida y aporta mucha información básica a las investigaciones.

Posteriormente a la elaboración de esta tesis, se ha continuado con el estudio de estos componentes y de otros presentes en el aceite de oliva virgen, en los que se han analizado las rutas moleculares y los genes implicados en esta acción preventiva diferencial, incluyéndose las vías relacionadas con el estrés oxidativo y la maquinaria antioxidante y las implicadas en la inflamación. Asimismo, se han realizado microarrays de expresión para determinar los pathways modificados. Todos estas investigaciones forman parte de otras tesis doctorales de las que alguna ya ha sido defendida y otras lo serán próximamente.

El crecimiento celular de la línea tumoral de mama MDA-MB-231 en los ensayos de proliferación tras la adición de un compuesto antioxidante, a pesar de no ser significativos, están en la línea de las teorías que apuntan a que el aporte de antioxidantes a individuos una vez que la enfermedad está presente en el organismo podría favorecer la proliferación del tumor (Lingying *y cols.*, 2015; Saeidnia *y cols.,* 2013; Sayin *y cols.,* 2014). Esta es una línea de trabajo de autores finlandeses y americanos están actualmente investigando, pero estimo que sería interesante profundizar más en estas investigaciones de las que podrían salir nuevos descubrimientos reveladores.

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ANEXOS

Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells.

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- Trabajo enviado a la revista "Food and Chemical Toxicology".
- Fecha de publicación: 28 de Enero de 2010
- Nº de citas (a 1 de Diciembre de 2015): 40
- SCImago Journal & Country Rank
  - Categoría perteneciente: Food Science
  - Índice SJR: 0,920
  - Posición: 36/241
  - Cuartil en la categoría: Q1
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Food Sciences and Technology
  - Índice de impacto: 2,895
  - Posición: 14/123
  - Cuartil en la categoría: Q1

Food and Chemical Toxicology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells

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ARTICLE INFO

Article history:
Received 1 October 2009
Accepted 28 January 2010
Available online xxxx

Keywords:
Squalene
Human breast cancer
Comet assay
Virgin olive oil
Cancer prevention

ABSTRACT

Until now, very little has been known about the antioxidant capacity of squalene and its effect on human breast tumourigenesis. In the present work, we investigated squalene's scavenging properties and its effect on cell proliferation, cell cycle profile, apoptosis, reactive oxygen species (ROS) level and oxidative DNA damage, using human breast cell lines. Our results showed that squalene neither possesses scavenging activity nor significantly alters cell proliferation rates, the cell cycle profile or cell apoptosis in human mammary epithelial cells (MCF10A), minimally invasive (MDA-MB-231) breast cancer cells, and highly invasive (MCF7) breast cancer cells. However, we found that squalene did exert the following effects on MCF10A epithelial cells in a dose-dependent manner: (a) it decreased intracellular ROS level, (b) it prevented H_2O_2-induced oxidative injury, and (c) it protected against oxidative DNA damage. Interestingly, squalene did not exert these effects on MCF7 and MDA-MB-231 cancer cells. Therefore, our data suggest that squalene, found in high amounts in virgin olive oils, could be partially responsible for the lower incidence of breast cancer in populations that consume the Mediterranean diet due to its protective activity against oxidative DNA damage in normal mammary cells.

1. Introduction

Olive oil is the principal source of fat in the Mediterranean diet (Pérez-Jiménez et al., 2005). Consumption of olive oil has been associated with a low incidence and prevalence of cancer, including breast cancer (Escrich et al., 2006; Owen et al., 2000a). It has been suggested that the lower risk of breast cancer associated with high olive oil consumption may be due to the presence of squalene (Newmark, 1997).

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane) is a triterpenic hydrocarbon present at high concentrations in shark liver and virgin olive oils. In virgin olive oil, it is the major hydrocarbon (more than 90%), with content ranging from 0.8 to 13 g/kg (Allouche et al., 2007; Cert et al., 2000; Owen et al., 2000b). Squalene, an intermediate metabolite in cholesterol metabolism, is considered a remarkable bioactive substance, with several interesting biological activities, including antioxidant and antitumour properties (Owen et al., 2000a). Indeed, *in vitro* experimental evidence indicates that this compound is a highly effective singlet oxygen scavenging agent (Saint-Leger et al., 1986) and a chemopreventive agent against chemically-induced cancer (Smith, 2000; Sotiroudis and Kyrtopoulos, 2008). Moreover, squalene is able to inhibit aberrant hyperproliferation, an event that precedes mammary tumourigenesis *in vivo* (Katdare et al., 1997), and to play a tumour-inhibiting role in animal models (Murakoshi et al., 1992; Newmark, 1997; Rao et al., 1998).

Despite evidence suggesting its anticarcinogenic and antitumour properties, there are no available studies on the effects of squalene either on human breast tumour or human mammary epithelial cells. Therefore, the aim of this paper was to investigate squalene's possible antioxidant effects and their relation to its antiproliferative capacity and oxidative DNA damage protection in both human breast cancer cell lines (MCF7 and MDA-MB-231)

Abbreviations: AAPH, 2,2′-azobis (2-methylpropionamidine) dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); CPT, camptothecin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; EMCCD, electron-multiplying charge coupled device; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; FL, fluorescein; HBSS, Hank's balanced salt solution; HuMEC, human mammary epithelial cells medium; LMA, low melting point agarose; MEM, minimum essential medium; NEAA, non-essential amino acids; NMA, normal melting point agarose; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffer saline; PI, propidium iodide; PMS, phenazine methosulphate; ROS, reactive oxygen species; RPMI, Roswell park memorial institute 1640 medium; TOC, α-tocopherol; XTT, (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate).

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doi:10.1016/j.fct.2010.01.031

and an immortalized non-tumourigenic human mammalian epithelial cell line (MCF10A). For this purpose, we studied squalene's scavenging activity and its effects on cell proliferation, cell cycle profile, apoptosis, intracellular oxidative stress and DNA oxidative damage.

2. Materials and methods

2.1. Materials

The following were purchased from Sigma–Aldrich Co. (St Louis, MO): Hepes Buffer; Sodium Pyruvate; non-essential amino acids mixture 100× (NEAA); 2′,7′-dichlorofluorescein diacetate (DCFH-DA); dimethyl sulfoxide (DMSO); 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium salt) purity ⩾90%; 5-methylphenazinium methyl sulfate; N-methylphenazonium methyl sulfate (PMS) purity ~98%; 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane (squalene CAS 111-02-4) purity ⩾98%; DL-all-rac-α-tocopherol (Vitamin E CAS 10191-41-0 (TOC)) purity ⩾96%; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity ⩾97%; 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) purity ~97%; 2,2-diphenyl-1-picrylhydrazyl (DPPH) purity ~90%; (S)-(+)-camptothecin (CAS 7689-03-4 (CPT)) purity ~95%; 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt tablets (CAS 30931-67-0 (ABTS)); PBS; HBSS. Minimum essential medium with Eagle's salts (MEM), foetal bovine serum (FBS) and phenol-red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express, HuMEC ready medium kit, fluorescein (FL) and propidium iodide (PI) were obtained from Invitrogen (Eugene, OR). $K_2S_2O_8$ (CAS 7727-21-1) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Culture plates were obtained from NUNC™ (Roskilde, Denmark). The PI/RNase Staining Buffer kit, FITC-conjugated Annexin V and Binding Buffer were obtained from BD Biosciences Pharmigen (San Diego, CA.). The comet assay kit was obtained from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD).

2.2. Estimation of radical scavenging activity by the DPPH test

The antioxidant activity of squalene against the stable radical DPPH was measured as previously reported (Brand-Williams et al., 1995) with some modifications. Briefly, 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of squalene in 96-well plates at 0.06, 0.13, 0.25, 0.5 and 1 mol of antioxidant/moles of DPPH. α-Tocopherol (TOC) was used as a standard antioxidant control and a sample without antioxidant was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min for 2 h in a microplate reader (TECAN, GENios Plus). Measurements were performed in triplicate.

The inhibition of the DPPH radical was calculated according to the following percentage of radical scavenging activity (% RSA) formula:

$$\%\ \mathrm{RSA} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance of the antioxidant at $t = 50$ min.

2.3. ABTS radical scavenging assay

ABTS cation radical scavenging activity was determined using a previously reported procedure (Re et al., 1999). ABTS radicals ($ABTS^{+\cdot}$) were obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{+\cdot}$ was diluted in ultrapure water until absorbance at 734 nm was 0.7 (±0.1). Squalene and Trolox™ (as antioxidant reference) were dissolved in ethanol to yield a 10 mM stock solution and diluted with ultrapure water to the assayed concentrations. Twenty microliters of each concentration of squalene, standard (Trolox™), blank (ultrapure water) or ethanol control (8%) were added to a 96-well plate. The reaction was initiated by the addition of 280 μL of $ABTS^{+\cdot}$. Absorbance readings were taken every 5 min at 30 °C for 2 h in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate.

The inhibition of $ABTS^{+\cdot}$ was calculated according to the percentage of radical scavenging activity (% RSA) described above (at $t = 30$ min).

2.4. ORAC assay

Squalene's peroxyl radical scavenging activity was measured by the $ORAC_{FL}$ assay as previously described (Prior et al., 2003). A stock solution of squalene was reconstituted in DMSO and then diluted in PBS. A stock solution of Trolox™, as reference antioxidant control, was also diluted in DMSO and diluted in PBS. The assay was carried out in 96-well plates with a final volume of 160 μL. Samples were run in triplicate. Fluorescein (48 nM) was mixed with various concentrations of squalene, standard (Trolox) or blank (PBS) containing at final volume 1% DMSO (v/v). Plates were incubated for 15 min at 37 °C. The assay was initiated by the addition of AAPH (100 mM), and fluorescence readings (Ex. λ_{485}/Em. λ_{520} nm) were taken every 5 min at 37 °C for 160 min in a microplate reader (TECAN GENios Plus). Final results were calculated based on the difference in the area under the fluorescence decay curve (AUC) between the blank and each sample. The AUC formula was:

$$\mathrm{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \cdots + f_{20}/f_0.$$

Results were expressed as micromolar Trolox™ equivalents (TE) calculated using the line equation from the standard curve:

$$\mathrm{TE} = (Y - b)/m$$

where Y is the net AUC ($AUC_{sample} - AUC_{control}$), m is the slope and b is the Y-intercept.

2.5. Cell culture

Highly invasive MDA-MB-231 human breast cancer cells (oestrogen and progesterone receptor-negative), minimally invasive MCF7 human breast cancer cells (oestrogen and progesterone receptor-positive), and immortalized non-tumourigenic human breast epithelial cells (MCF10A), were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Breast tumour cells (MCF7 and MDA-MB-231) were grown as monolayer cultures in MEM supplemented with 10% FBS, 1% Hepes Buffer, 1% Sodium Pyruvate and 1% NEAA. Human mammary epithelial cells (MCF10A) were cultivated in HuMEC Ready Medium. All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured using TrypLE Express solution (Invitrogen). Cells in the exponential growth phase were used for all experiments.

2.6. XTT cell proliferation assay

Cell proliferation, measured as the cellular growth of treated cells *versus* untreated controls, was carried out using an XTT-based assay according to Scudiero et al. (1988) with some modifications. Briefly, cells were seeded into 96-well culture plates. After overnight incubation to allow cell attachment, medium was removed and replaced with fresh medium containing squalene at a series of concentrations and plates were incubated for 24, 48 or 120 h followed by a 6 day proliferation period (incubation with fresh medium). At these three time points, plates were incubated with XTT in RPMI without Phenol-Red for 3 h and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

$$\%\ \text{viable cells} = [(A_{\text{treated cells}})/(A_{\text{control}})] \times 100$$

where A is the difference in absorbance between optical density units ($A = OD_{450} - OD_{620}$)

All measurements were performed in triplicate and each experiment was repeated at least three times.

2.7. Cell cycle assay

Cells were seeded in 12-well culture plates and treated with different doses of squalene for 24 h. After incubation, cells were fixed with cold 70% ethanol and stored at −20 °C for at least 24 h. Subsequent to propidium iodide labeling (PI/RNase Staining Buffer), cells were analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). The FlowJo program (v5.7.2) was used to calculate the percentage of cells in G_0/G_1, S and G_2/M phases. Each experiment was repeated at least three independent times.

2.8. Analysis of apoptosis

The percentage of apoptotic cells was determined using a double staining assay with FITC-conjugated Annexin V and propidium iodide (PI). Briefly, after cell exposure to squalene for 24 h, cells were harvested, washed twice in cold PBS and resuspended in 100 μL Annexin Binding Buffer. Cells were stained with 5 μL Annexin V-FITC and 1 μL PI solution, gently vortexed and incubated for 15 min at room temperature in the dark before flow cytometric analysis. As a positive control, cells were treated with 1 μM camptothecin (CPT). Each experiment was repeated at least three independent times.

2.9. Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured after 24 h of treatment with different doses of squalene, or TOC as a positive control, using the cell-permeable fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described (Wang and Joseph, 1999) with some modifications. In brief, after incubation, cells were washed two times with Hank's buffered salt solution (HBSS) and incubated with fresh DCFH-DA (100 μM) for 30 min at 37 °C in 5% CO_2. Cells were then washed twice and read in a plate reader for 30 min (Ex. λ_{485}/Em. λ_{535}, Gain 60). The intracellular ROS level percentage was calculated as follows:

$$F = [(Ft_{30} - Ft_0)/Ft_0] \times 100$$

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where Ft_0 is the fluorescence at $t = 0$ min and Ft_{30} the fluorescence at $t = 30$ min.

It has been reported that the addition of H_2O_2 increases oxidative stress in cultured cells (Lee et al., 2006). Therefore, in order to evaluate the protective capacity of squalene against induced oxidative stress, H_2O_2 at 500 μM was added 30 min before the fluorescence quantification.

All tests were run in triplicate for each experimental condition and each experiment was repeated at least three times. All experiments were conducted using iron-free mediums (MEM and HuMEC).

2.10. Alkaline single-cell gel electrophoresis (comet assay)

At 24 h, cells treated with squalene were scraped into 12-well culture plates, washed twice (300*g* 10 min, 4 °C) with cold 1X PBS (Ca^{2+}/Mg^{2+} free) and then resuspended in 1 ml of cold 1X PBS. In order to evaluate squalene's ability to protect against oxidative DNA damage, cell suspensions were exposed for 10 min to 50 μM H_2O_2 at 4 °C. After that, cells were washed twice and frozen in FBS-DMSO (90:10, v/v) at −80 °C until the comet assay procedure.

DNA single strand break by alkaline microgel electrophoresis was performed according to Singh et al. (1988) with some modifications. Cells were thawed in a bath at 37 °C, centrifuged (300*g* 10 min, 4 °C) in cold MEM with 25% FBS and resuspended in cold 1X PBS to a density of 1.65×10^5 cells/ml. Cells were then suspended in melted and cooled (at 40 °C) low melting point agarose (LMA). Cell suspensions (50 μL) were spread over a sample area of pre-warmed 1% normal melting point agarose (NMA) precoated CometSlide™ slides (Trevigen, Inc.). After 15 min at 4 °C in the dark, slides were immersed in cold Lysis Solution (Trevigen, Inc.) at 4 °C for 30 min to dissolve lipids and proteins. In order to separate the two DNA strands, slides were then immersed in fresh Alkaline Solution (pH > 13) for 30 min at room temperature in the dark. Electrophoresis was performed in a refrigerated black electrophoresis tank containing cold Alkaline Electrophoresis Solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 25 V (1 V/cm) and 300 mA for 40 min. The slides were washed twice with distilled water for 10 min and neutralized with 10 mM Tris–HCl, pH 7.5 for 5 min, followed by immersion in 70% ethanol for 5 min, and air-dried overnight at room temperature. Slides were stained with Sybr green before scoring.

2.11. Slide scoring and analysis

DNA strand breaks were examined in a fluorescence microscope (Zeiss Axiovert 200) equipped with Luca EMCCD camera (Andor Technology, Belfast, UK) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). Fifty cell images were randomly characterized per sample using 20× magnification. Relative fluorescence between head and tail through the olive tail moment (Olive_TM) was used to determine DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

$$\text{Olive\_TM} = [(\text{tail(mean)} - \text{head(mean)}) \times \text{tail(\% DNA)}]/100.$$

2.12. Statistical analysis

Results are presented as mean (±SEM), except for cell proliferation results. For this assay, results are presented as mean (±SD). Results are expressed as a percentage relative to the control, which was defined as 100%. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test. Values of $p < 0.05$ were considered significant. STATGRAPHICS Plus 5.1 statistical software (Statpoint Technologies, Inc., Warrenton, Virginia, USA) was used for the statistical analysis.

3. Results

3.1. Antioxidant activity

Radical scavenging capacity was determined using DPPH, ABTS and ORAC assays.

The antiradical activity of squalene, as measured by scavenging activity in the DPPH radical assay (Fig. 1A) or in the ABTS cationic radical assay (Fig. 2A), indicates that squalene at up to 10 mol ratio [mole antioxidant/mole DPPH] does not possess antiradical activity, nor does it possess such activity at up to 800 μM. TOC

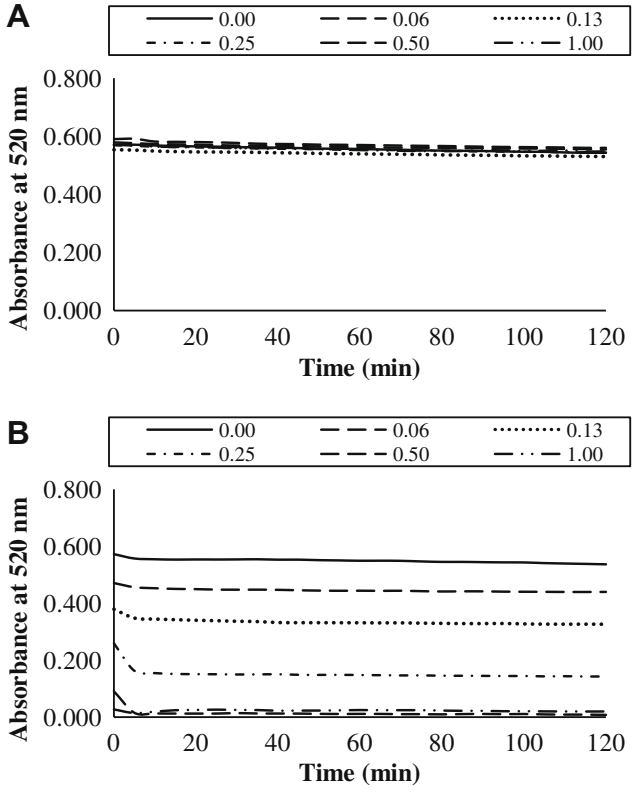

Fig. 1. Radical scavenging activity was measured by the reduction of the DPPH radical (100 μM) by ethanolic solutions of squalene (A) or α-tocopherol (TOC) (B) for a period of 120 min. The mole ratios [mole antioxidant/mole DPPH] assayed were between 0.06 and 1. The relative radical scavenging activity (RSA) of squalene at 50 min was ⩽5% even at higher ratios (2.50, 5.00 and 10.00) (data not shown) while the RSA of Trolox™ was 50% at 0.16 mol ratio.

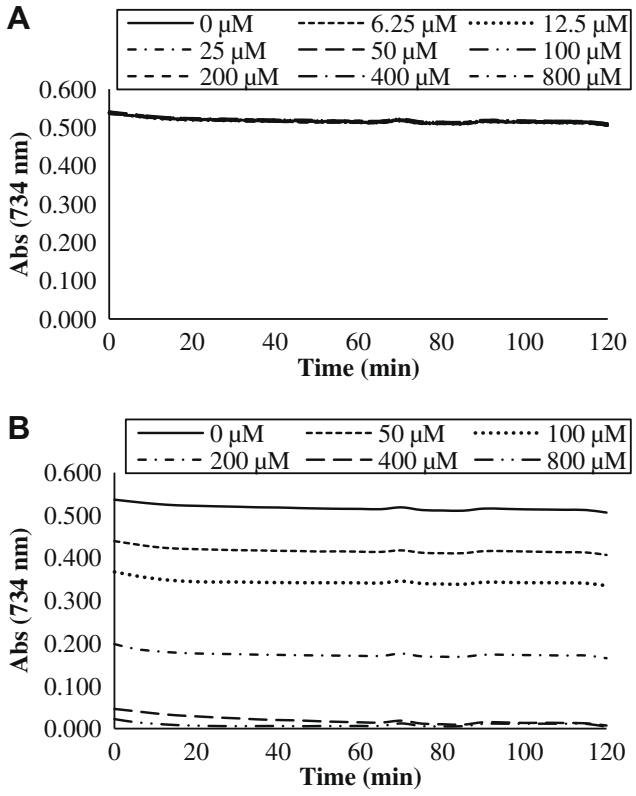

Fig. 2. ABTS radical cation ($ABTS^{+\cdot}$) antioxidant activity of ethanolic solutions of squalene (A) or Trolox™ (B) up to 800 μM were measured by decolorization of $ABTS^{+\cdot}$ for 120 min. Relative radical scavenging activity (RSA) for all assayed concentrations of squalene at 30 min was <4% while the RSA of Trolox™ reached 50% at 143.57 μM.

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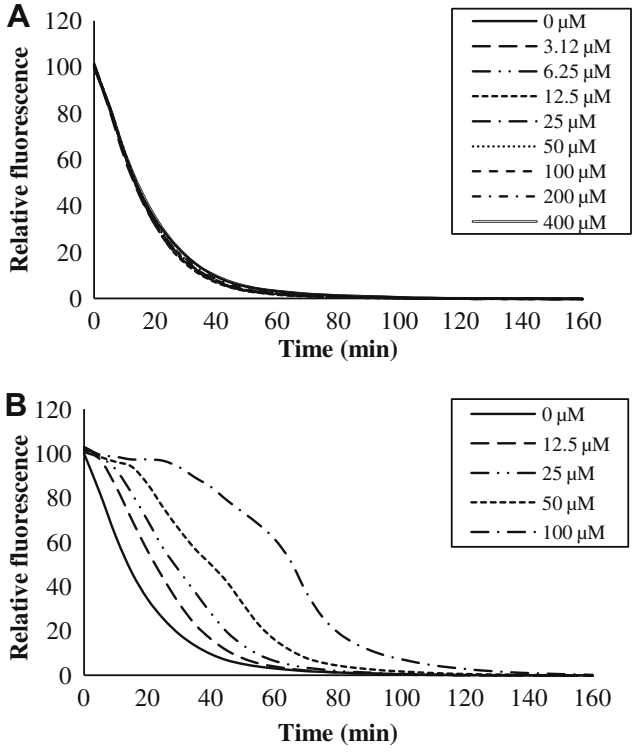

Fig. 3. Peroxyl antiradical activity was performed by the ORACFL assay on a range of concentrations of squalene from 3.12 to 400 μM (A) or Trolox™ from 12.5 to 100 μM (B) for 160 min. Results show how squalene failed to reduce the AAPH-induced peroxyl radical at all assayed concentrations compared with Trolox™. TE for any one of the squalene concentrations assayed was close to zero, indicating the absence of peroxyl scavenging activity (data not shown).

and Trolox were used as antioxidant standard controls for the DPPH (Fig. 1B) and ABTS (Fig. 2B) assays, respectively.

Squalene's peroxyl radical scavenging activity, measured by the $ORAC_{FL}$ assay, shows the absence of a protective effect against AAPH-induced peroxyl radical activity (Fig. 3A), as compared with Trolox's antiradical activity (Fig. 3B).

3.2. Effect of squalene on cell proliferation

To observe the effect of squalene on both human breast cancer cell lines (MCF7 and MDA-MB-231) and on the immortalized non-tumourigenic human mammary epithelial cell line (MCF10A), cells were treated with various concentrations of squalene ranging from 3.12 to 50 μM for 24, 48 and 120 h. Squalene had no significant effect on the cell proliferation rates of both MCF7 and MCF10A cells (Fig. 4A and C). Unexpectedly, a slight increase in MDA-MB-231 cell proliferation was observed, although it was not statistically significant (Fig. 4B).

3.3. Effect of squalene on the cell cycle

A flow cytometry assay after PI staining allows cell cycle analysis. MCF7, MDA-MB-231 and MCF10A cells were treated with 12.5, 50 and 200 μM squalene to evaluate interference with the cell cycle. The results revealed that after 24 h of squalene treatment, no significant effect was observed on the cell cycle in any one of the three cell lines tested (data not shown).

3.4. Effect of squalene on apoptosis

The extent of apoptosis was assessed by flow cytometry analysis following 24 h exposure of cells to different doses of squalene (12.5, 50 and 200 μM). Apoptosis was detected using Annexin-V-FITC (AV) and PI labeling. The percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells to all cells (apoptotic and whole cells). Flow cytometric analysis revealed that treatment with squalene for 24 h did not induce apoptosis in human breast epithelial cells (MCF10A), nor did it induce apoptosis in human breast cancer cells (MCF7, MDA-MB-231), when compared with controls (data not shown).

3.5. Effect of squalene on intracellular ROS production

Measurement of intracellular ROS levels using the DCFH-DA probe showed a significant and dose-dependent decrease in ROS levels in MCF10A cells after 24 h treatment with increasing concentrations of squalene from 3.12 to 50 μM. At the highest concentration (50 μM), squalene reduced ROS production by more

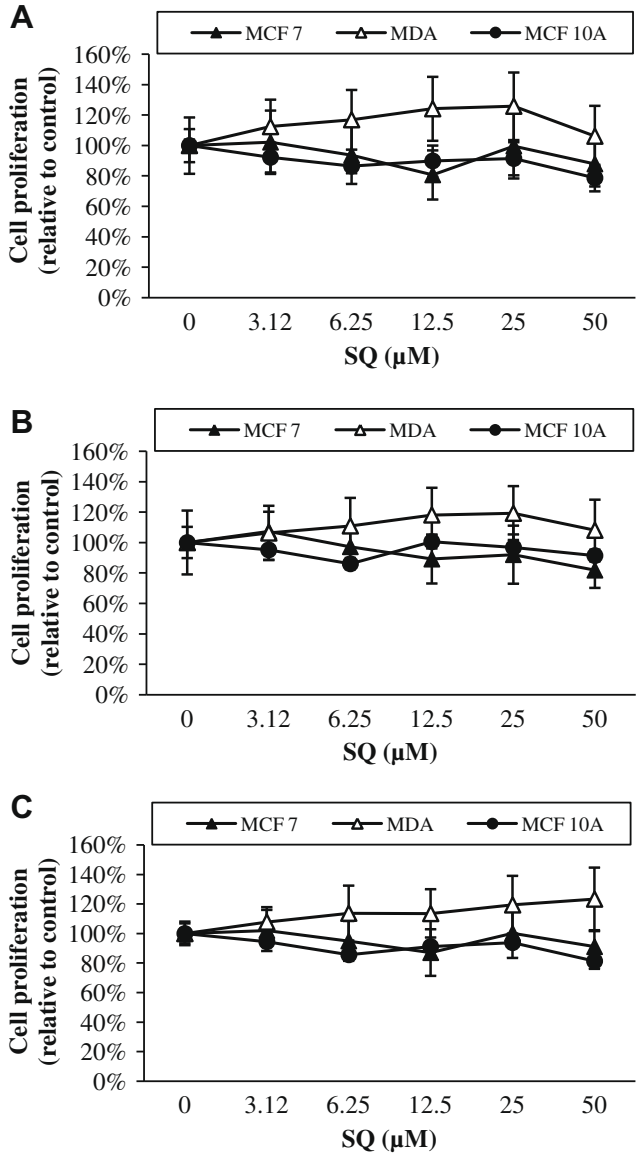

Fig. 4. *In vitro* cell proliferation of MCF7, MDAMB-231 and MCF10A cell lines after squalene (SQ) treatment was determined by colorimetric XTT-based assay at 24 h (A), 48 h (B) and 120 h (C). Although MDA-MB-231 showed a slight increase in cell proliferation, no difference at $p < 0.05$ was detected in any cell line. Higher concentrations of squalene up to 400 μM showed similar effects (data not shown). Values represent the mean ± SD of three independent experiments and are expressed as a percentage of the control, which was defined as 100%.

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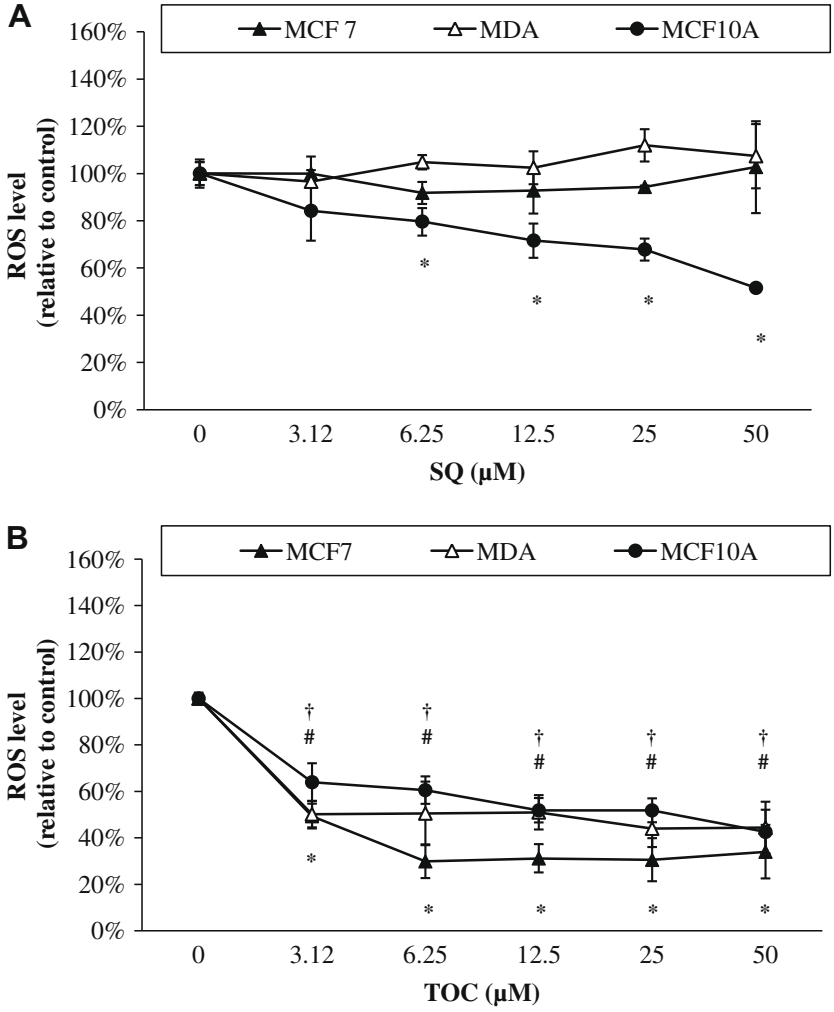

Fig. 5. Intracellular ROS levels were measured by DCFH-DA probe in MCF7, MDA-MB-231 and MCF10A cell lines after treatment with squalene (SQ) (A) or α-tocopherol (TOC) (B) for 24 h. Squalene significantly decreased intracellular ROS levels, in a dose-dependent manner, in MCF10A cells but not in MCF7 and MDA-MB-231 cells. TOC significantly decreased the intracellular ROS level in a dose-dependent manner in both breast cancer and mammary epithelial cell lines. Results are displayed as mean ± SEM for three independent experiments. Significant differences were determined relative to the control, which was considered to be 100%, at $p < 0.05$ († MCF7, # MDA-MB-231, * MCF10A) using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

than 50% compared with the untreated control. Interestingly, squalene did not change intracellular ROS levels for either of the human breast cancer cell lines tested (MCF7, MDA-MB-231) (Fig. 5A).

TOC, used as an antioxidant reference compound, exhibited a significant decrease in ROS levels, with a greater than 50% reduction compared with untreated cells in each of the cell lines assayed (MCF10A, MCF7 and MDA-MB-231) (Fig. 5B).

To induce intracellular oxidative stress, H_2O_2 was added before fluorescence measurement. H_2O_2 induces oxidative injury on MCF7, MDA-MB-231 and MCF-10A cells *in vitro* (data not shown). In order to investigate the *in vitro* preventive effect of squalene against H_2O_2 oxidative injury, intracellular ROS levels were measured in cells previously treated with squalene for 24 h at increasing concentrations from 3.12 to 50 μM. Interestingly, whereas squalene did not prevent oxidative stress injury in MCF7 and MDA-MB-231 breast tumour cells, MCF10A epithelial cells showed a significant decrease (up to 60% reduction) in intracellular ROS levels, in a dose-dependent manner (Fig. 6A). In contrast, at even the lowest concentration (3.12 μM), TOC was able to reduce oxidative injury in both the tumour cells and the epithelial cells (Fig. 6B). However, squalene was more effective at reducing oxidative injury to MCF10A epithelial cells than TOC.

3.6. Preventive effect of squalene against oxidative DNA damage

We measured the preventive effect of squalene against H_2O_2-induced DNA damage in MCF10A, MCF7 and MDA-MB-231 cells using alkaline single-cell gel electrophoresis (comet assay). Squalene's potential to reduce oxidative DNA damage was expressed as Olive_TM. Olive_TM incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail) (Singh et al., 1988).

Pre-incubation of MCF10A cells with increasing concentrations of squalene for 24 h exerted a significant, dose-dependent preventive effect against H_2O_2-induced DNA damage ($p < 0.001$) (Fig. 7A). However, squalene did not show any preventive effect against the formation of single-strand breaks in MCF7 and MDA-MB-231 tumour cells. An increase in oxidative DNA damage was observed in the MDA-MB-231 cell line, but this increase was not statistically significant ($p = 0.23$) (Fig. 7B).

4. Discussion

Case control studies have shown an inverse correlation between olive oil consumption and the incidence of breast cancer (Escrich

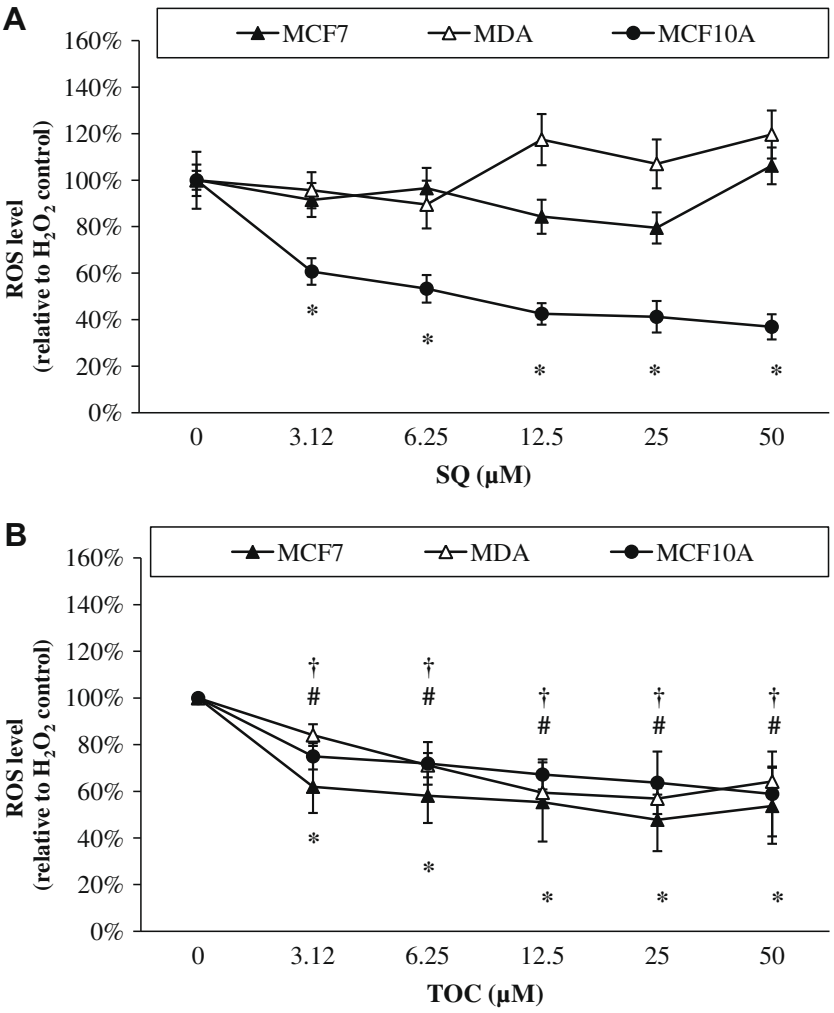

Fig. 6. The capacity of squalene (SQ) (A) or α-tocopherol (TOC), as antioxidant control (B), to reduce an oxidative burst induced by the addition of H_2O_2 (500 μM) on MCF7, MDA-MB-231 or MCF10A cell lines was measured by DCFH-DA probe. Squalene prevented the oxidative injury induced in MCF10A cells, in a dose-dependent manner, but this preventive effect was not observed in MCF7 and MDA-MB-231 cells. Interestingly, TOC significantly reduced oxidative injury in all three cell lines, even at the lowest concentration (3.12 μM). Results are expressed as mean ± SEM for three independent assays. Significant differences were determined relative to the positive control (treated with H_2O_2), which was defined as 100%, at $p < 0.05$ († MCF7, # MDA-MB-231, * MCF10A) using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

et al., 2006; Owen et al., 2000a). It has been suggested that the lower risk of breast cancer associated with olive oil consumption may be due to squalene (Newmark, 1997), a minor compound present in virgin olive oil in quantities as high as 13,000 mg/kg (Allouche et al., 2007). The average daily dietary intake of squalene in Mediterranean countries is in the range of 200–400 mg/day (Sotiroudis and Kyrtopoulos, 2008).

Experiments in rodents suggest that squalene exhibits antitumour activity against skin, colon, sarcoma and lung cancer (Murakoshi et al., 1992; Ohkuma et al., 1983; Rao et al., 1998; Smith et al., 1998). It has also been shown that squalene inhibits the *in vitro* growth of neuroblastoma cells (Das et al., 2003). The mechanism proposed to explain the activity of squalene is based on its strong inhibitory action on beta-hydroxy-beta-methylglutaryl-CoA reductase catalytic activity *in vivo*. This activity affects Ras p21 farnesylation, signal transduction and cellular proliferation (Newmark, 1997). Nevertheless, very little is known about the effect of squalene on human breast tumourigenesis.

Our data suggest that squalene treatment did not exert any significant influence on MCF10A, MCF7 and MDA-MB-231 cell proliferation. (We did observe a slight increase in MDA-MB-231 cell proliferation, but the increase was not statistically significant.) Das et al. (2008) have hypothesized that squalene might support the growth of a small fraction of tumour stem cell-like cells having a very high tumourigenic capacity after cisplatin treatment. In addition, it has been reported that at low-doses, certain antioxidants can stimulate the growth of some types of cancer. For example, vitamin C was found to stimulate the growth of both human parotid carcinoma cells and human leukemic cells *in vitro* (Prasad et al., 2001), and vitamin E was found to enhance the growth of various prostate cancer cell lines (Bureyko et al., 2009). Beta-carotene, an antioxidant structurally similar to squalene, increased the incidence of lung cancer among male heavy cigarette smokers (Paolini et al., 2003). Future work is needed to clarify the mechanisms of stimulation of cancer cell growth by some antioxidants.

In the present study, the effect of squalene on the cell cycle profile and cell apoptosis was also studied. Incubation of MCF10A, MCF7 and MDA-MB-231 cells for 24 h with squalene did not alter the cell cycle parameters, nor did it induce cell apoptosis. Overall, these results suggest that squalene does not induce breast tumour cell death and may be ineffective once a breast tumour is established.

Squalene has long been considered to be an antioxidant exerting anticarcinogenic activity by enhancing cellular antioxidant status (Hashim et al., 2005; Murakoshi et al., 1992; Smith, 2000). However, very little is known about its scavenging capacity. Our

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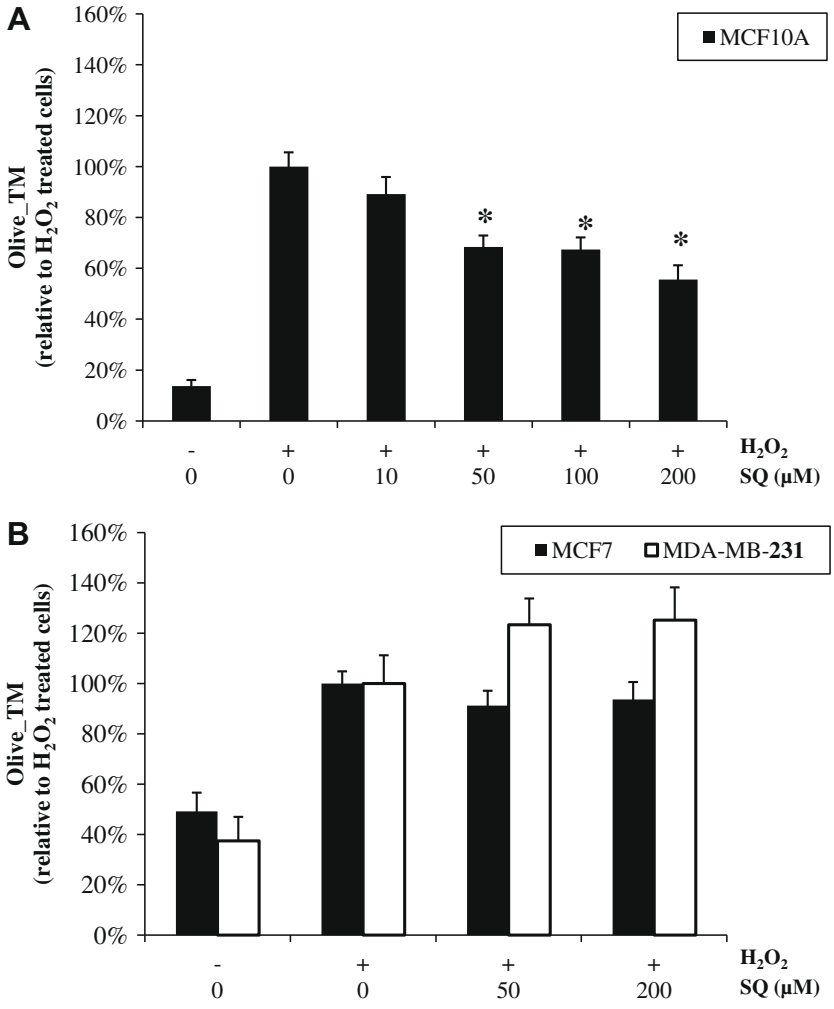

Fig. 7. A comet assay was performed in order to determine the preventive effect of squalene (SQ) in DNA oxidative damage in the MCF10 cell line (A) or MCF7 and MBA-MB-231 cell lines (B). Squalene reduces single-strand breaks, in a dose-dependent manner, in MCF10A cells, but not in MCF7 and MBA-MB-231 cells. Olive_TM values are represented by mean ± SEM for three independent assays. Significant differences were determined relative to the positive control (treated only with H_2O_2), which was defined as 100%, at $^*p < 0.05$ using one-way analysis of variance (ANOVA) followed by Fisher's LSD test.

results showed that squalene did not exert antioxidant activity against DPPH stable radicals, ABTS cation radicals or APPH-induced peroxyl radicals, even at high concentration (10.00 mol ratio, 800 μM and 400 μM, respectively) (Figs. 1–3). Concomitantly, it has also been reported that squalene reacts very poorly with DPPH (EC50 at 980 mol ratio) (Psomiadou and Tsimidou, 1999), and has a very low ORAC value (0.00062), suggesting that squalene's antioxidant activity is extremely low (Tikekar et al., 2008). Squalene's lack of ABTS scavenging capacity has not been described previously. In contrast, Kohno et al. (1995) reported squalene to be a singlet oxygen quencher capable of protecting the skin from UV radiation. The plasma membrane is permeable to squalene, enabling squalene to move inside the lipid bilayer and act as a quencher barrier for singlet oxygen, consequently preventing intracellular oxidative damage. Differential squalene permeability between tumour and non-tumour mammary cells could provide a possible explanation for these contrasting results, but the evidence at this point is inconclusive.

Squalene has been found to exert protective activity against several cancers associated with specific carcinogens, including azoxymethane-induced colon cancer (Rao et al., 1998) and nicotine-derived nitrosaminoketone-induced lung carcinogenesis (Smith et al., 1998). In the present study, the DCFH-DA assay indicated that squalene has differential antioxidant activity in human breast cells, since it significantly decreased, in a dose-dependent manner, intracellular ROS levels in MCF10A cells (57.71% at 50 μM) but not in MCF7 or MDA-MB-231 cells (104.87% and 99.92% at 50 μM, respectively). This selective effect of squalene was also described by Das et al. (2008), although in other cell types. These authors found that squalene decreased ROS levels in bone marrow cells but not in neuroblastoma cells (SK-N-BE tumour cell line). In contrast, the well-known antioxidant TOC, used as the reference in the present study, did not show this selective effect; it reduced the oxidative stress by 58.84%, 53.78% and 64.20% at 50 μM in MCF10A, MCF7 and MDA-MB-231 cells, respectively.

Consequently, our results suggest that breast cancer cells and breast epithelial cells have the same response to TOC treatment, but that squalene acts as an antioxidant only on mammary epithelial cells. Taking into account that the mechanism of such selective antioxidant sensitivity is unknown, we may consider the following possibilities: (i) it is possible that squalene selectively increases glutathione (GSH) level in normal cells but not in breast cancer cells, a phenomenon described as the "GSH paradox" (Das et al., 2003); (ii) the selective sensitivity might be related to differences in cellular uptake and accumulation of squalene, or the status of the mevalonate pathway (Das et al., 2008); (iii) the selective sensitivity might be related to the differential regulation of antioxidant systems in normal *versus* tumour cells (Klauning and Kamendulis,

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2004). Tumour cells may be in an environment of continuous oxidative stress, in which antioxidants may act as pro-oxidants. In fact, in the present study we found that breast cancer cells have higher intracellular ROS levels than non-tumourigenic mammary epithelial cells (data not shown). Further studies should be done to address these topics.

Oxidative stress may result in an increase in oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant system. It is known that a chronic increase in ROS and oxidant stress may induce cancer (Ambrosone, 2000). Reactive oxygen species can damage cellular macromolecules, including DNA, and this damage is directly responsible for carcinogenesis. Thus, it has been shown that neoplastic transformation is associated with an increase in the basal oxidant level. In fact, serum markers for oxidative DNA damage have been shown to increase in women diagnosed with breast cancer (Musarrat et al., 1996). Considering that oxidative stress is involved in the pathophysiology of all cancers (Visioli et al., 2004), the prevention of oxidative stress on mammary cells could be a suitable way to prevent breast cancer development.

In the present work, we found that H_2O_2-induced oxidative injury increases ROS levels in both human mammary epithelial cells and breast cancer cells as measured by the DCFH-DA assay (data not shown). Interestingly, squalene was able to prevent, in a dose-dependent manner, induced oxidative injury in human breast epithelial cells but not in human breast cancer cells. It has been postulated that neoplastic transformation is associated with an increase in the basal level of oxidant stress; therefore, it may be presumed that squalene might help to prevent human breast cancer by reducing oxidative stress on mammary epithelial cells.

The comet assay provides information about DNA damage through quantification of single-strand breaks. We used this technique to determine the protective effect of squalene against DNA damage induced by H_2O_2 oxidative injury. As observed in the DCFH-DA assay, squalene exhibited selective activity according to the cell line. It reduced oxidative DNA damage in a dose-dependent manner in MCF10A mammary epithelial cells, but failed to reduce oxidative injury in MCF7 and MDA-MB-231 breast cancer cells. In the latter, injury was even slightly increased after squalene treatment.

Therefore, these results suggest that squalene might be helpful in human breast cancer prevention, but not treatment, based on two hypotheses: (i) it reduces oxidative stress by decreasing ROS levels in mammary epithelial cells, and (ii) it selectively protects against oxidative DNA damage in mammary epithelial cells. In addition, these findings could support the hypothesis that high squalene intake could contribute to the lower incidence of breast cancer in Mediterranean populations. It is important to note that adipose tissue contains exceptionally high concentrations of squalene relative to other tissues (Tilvis et al., 1982). Considering that mammary glands include much adipose tissue, it may be presumed that squalene levels could also be high in breast tissues.

In summary, we found that mammary epithelial cells and breast tumour cells differed in their responses to squalene treatment. Our results indicated that squalene reduces *in vitro* ROS levels and protects against oxidative DNA damage in human mammary epithelial cells but not in breast tumour cells. Therefore, squalene, present at high concentrations in virgin olive oil, might contribute to the preventive effect of olive oil against human breast cancer by inhibiting oxidative stress. Moreover, our *in vitro* results suggest that squalene may play a role in the prevention of human breast cancer, but is probably ineffective once breast tumours are established. Nevertheless, extreme caution should be applied in the extrapolation of *in vitro* results to potential human effects. Further studies are needed to confirm both the *in vivo* protective capacity of squalene and the differential mechanism of action on normal *versus* breast cancer cells suggested by the present study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was supported by the "Ministerio de Ciencia e Innovación" (RTA2008-00066-C03-03); "Instituto Andaluz de Biotecnología" (BIOÁNDALUS 08/22/L5.3); and; "Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud" (CEAS).

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Please cite this article in press as: Warleta, F., et al. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Food Chem. Toxicol. (2010), doi:10.1016/j.fct.2010.01.031

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Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells

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- Trabajo enviado a la revista “Nutrients”.
- Fecha de publicación: Octubre 2011
- Nº de citas (a 1 de Diciembre de 2015): 31
- SCImago Journal & Country Rank
  - Categoría perteneciente: Food Science
  - Índice SJR: 1,174
  - Posición: 24/241
  - Cuartil en la categoría: Q1
- Thomson Reuters Journal Citation Reports
  - Categoría perteneciente: Nutrición y Dietética
  - Índice de Impacto: 3.270
  - Posición: 21/77
  - Cuartil en la categoría: Q2

Nutrients **2011**, *3*, 839-857; doi:10.3390/nu3100839

OPEN ACCESS

nutrients

ISSN 2072-6643

www.mdpi.com/journal/nutrients

Article

Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells

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Received: 27 July 2011; in revised form: 8 September 2011 / Accepted: 14 September 2011 / Published: 13 October 2011

Abstract: Over recent years, several studies have related olive oil ingestion to a low incidence of several diseases, including breast cancer. Hydroxytyrosol and tyrosol are two of the major phenols present in virgin olive oils. Despite the fact that they have been linked to cancer prevention, there is no evidence that clarifies their effect in human breast tumor and non-tumor cells. In the present work, we present hydroxytyrosol and tyrosol's effects in human breast cell lines. Our results show that hydroxytyrosol acts as a more efficient free radical scavenger than tyrosol, but both fail to affect cell proliferation rates, cell cycle profile or cell apoptosis in human mammary epithelial cells (MCF10A) or breast cancer cells (MDA-MB-231 and MCF7). We found that hydroxytyrosol decreases the intracellular reactive oxygen species (ROS) level in MCF10A cells but not in MCF7 or MDA-MB-231 cells while very high amounts of tyrosol is needed to decrease the ROS level in MCF10A cells. Interestingly, hydroxytyrosol prevents oxidative DNA damage in the three breast cell lines. Therefore, our data suggest that simple phenol hydroxytyrosol could contribute to a lower incidence of breast cancer in populations that consume virgin

olive oil due to its antioxidant activity and its protection against oxidative DNA damage in mammary cells.

Keywords: breast cancer; Mediterranean diet; olive oil minor compounds; hydroxytyrosol; tyrosol; phenols; oxidative stress; reactive oxygen species; DNA damage

1. Introduction

Olive oil is the major source of fats in the Mediterranean diet and is considered to be responsible for the health benefits associated with this diet. In fact, it has been demonstrated that people who consume virgin olive oil (VOO) present a lower incidence of several cancers, including breast cancer [1]. This effect has previously been attributed to the high content of monounsaturated fatty acids. However, more recently, the importance of the minor constituents of olive oil has been considered [2]. Over the last five decades, several publications have firmly established that ingestion of small quantities of certain compounds isolated from plants can lower the risk of cancer in mammals exposed to carcinogens, including polyphenols [3].

VOO contains relatively high amounts of minor compounds compared to other oils (refined olive oil or seed oils). Among these, phenolic compounds are present at levels between 200 and 1500 mg/kg [4] depending on the olive tree variety, climatic and agronomic conditions, degree of maturation at harvest, and the manufacturing process [4]. At present, there are many studies reporting biological activities *in vitro*, *in vivo* and in clinical assays of phenolic compounds naturally present in VOO. Between them, anti-inflammatory, cardioprotective antioxidant and chemopreventive effects in breast and other types of cancers have been defined [5]. The major phenols identified in olive oils include the simple phenols hydroxytyrosol (HT) and tyrosol (TY), secoiridoids and lignans [2]. The concentration of TY is always higher than of HT [6]. Hydrolysis of secoiridoid during olive oil storage results in the formation of HT and TY [7].

It has been well established that HT is a potent antioxidant because of its marked antioxidant activity, its ability to scavenge oxygen and nitrogen free radicals, to inhibit Low Density Lipoprotein (LDL) oxidation, platelet aggregation and endothelial cell activation and its protection against DNA damage [2,8]. HT was able to reduce the synthesis of prostaglandin E2 blocking the transcription of COX-2 and 5-lipooxygenase, thereby reducing the chronic influence associated with diseases such as cancer [9]. TY has been described as exerting a weak antioxidant activity, although it is able to scavenge peroxynitrite and superoxide radicals, inhibit LDL oxidation in Caco2 cells and inhibit LPS-induced cytokines release from human monocytes [10,11].

It has been suggested that HT and TY compounds might have preventive activity against breast cancer, but, at present, the exact role played by these phenols in breast cancer prevention is still unknown. In this sense, despite epidemiological evidence, *in vitro* experiments have not been conducted to check if there are different effects of the simple phenols HT and TY between human breast cancer cells and human breast non-cancer cells.

The present study attempts to provide new insights into the antioxidant capacity of HT and TY and the *in vitro* effects on proliferation, cell cycle progression, apoptosis, reactive oxygen species (ROS)

production and oxidative DNA damage in the human breast epithelial MCF10A cell line and the human breast MCF7 and MDA-MB-231 cancer cell lines.

2. Experimental Section

2.1. Chemicals and Materials

The following were purchased from Sigma-Aldrich Co. (St Louis, MO, USA): Hepes Buffer; Sodium Pyruvate; Non-Essential Amino Acids mixture 100× (NEAA); 2′,7′-dichlorofluorescein diacetate (DCFH-DA); Dimethyl sulfoxide (DMSO); 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT sodium salt) purity ≥90%; *N*-Methylphenazonium methyl sulfate (PMS) purity ~98%; 2-hydroxyphenyl ethanol (Tyrosol, CAS 501-94-0 (TY)) purity 98%; DL-all-rac-α-Tocopherol (Vitamin E, CAS 10191-41-0 (TOC)) purity ≥96%; 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity ≥97%; 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) purity ~97%; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) purity ~90%, (*S*)-(+)-camptothecin (CAS 7689-03-4 (CPT)) purity ~95%; 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt tablets (CAS 30931-67-0 (ABTS)); PBS; HBSS. 2-(3,4-dihydroxyphenyl) ethanol (Hydroxytyrosol, CAS 10597-60-1 (HY)) purity ≥98% was obtained from Cayman Chemical (Ann Arbor, MI, USA). Minimum essential medium with Eagle's salts (MEM), Fetal Bovine Serum (FBS) and Phenol-Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express, HuMEC Ready Medium kit and Fluorescein (FL) were obtained from Invitrogen (Eugene, OR, USA). $K_2S_2O_8$ (CAS 7727-21-1) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Culture plates were obtained from NUNC™ (Roskilde, Denmark). The PI/RNase Staining Buffer kit, FITC-conjugated Annexin V and Binding Buffer were obtained from BD Biosciences Pharmigen (San Diego, CA, USA). The Comet assay kit was obtained from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD, USA).

2.2. DPPH Assay

The antioxidant activity of HT and TY against the stable radical DPPH was measured as previously reported [12] with some modifications. Briefly, 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of HT or TY in 96-well plates at 0.06, 0.13, 0.25, 0.5 and 1 (moles of antioxidant/moles of DPPH). (±)-α-tocopherol (TOC) was used as a positive control and a sample without antioxidant was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min for 2 h in a microplate reader (TECAN, GENios Plus). Measurements were performed in triplicate.

The inhibition of the DPPH radical was calculated according to the following percentage of Radical Scavenging Activity (% RSA) formula:

$$\% \text{ RSA} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 50 min.

2.3. ABTS Assay

ABTS cation radical scavenging activity was determined using a previously reported procedure [13]. ABTS radicals ($ABTS^{\bullet+}$) were obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{\bullet+}$ was diluted in ultrapure water until absorbance at 734 nm was 0.7 (±0.1). HT, TY and Trolox™ (TR) (as antioxidant reference) was dissolved in ethanol to yield a 10 mM stock solution and diluted with ultrapure water to the assayed concentrations. Twenty microliters of each concentration of HT, TY, standard (TR), blank (ultrapure water) or ethanol control (8%) were added to a 96-well plate. The reaction was initiated by the addition of 280 μL of $ABTS^{\bullet+}$. Absorbance readings were taken every 5 min at 30 °C for 2 h in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate.

The inhibition of $ABTS^{\bullet+}$ was calculated according to the percentage of Radical Scavenging Activity (% RSA) described above (at t = 30 min).

2.4. ORAC Assay

Peroxyl radical scavenging activity was measured by the $ORAC_{FL}$ assay as previously described [14]. A stock solution of HT or TY were reconstituted in DMSO and then diluted in PBS. A stock solution of TR, as antioxidant standard, was also diluted in DMSO and diluted in PBS. The assay was carried out in 96-well plates with a final volume of 160 μL. Samples were run in triplicate. Fluorescein (48 nM) was mixed with various concentrations of SQ, standard (TR) or blank (PBS) containing at final volume 1% (v/v) DMSO. Plates were incubated for 15 min at 37 °C. The assay was initiated by the addition of AAPH (100 mM) and fluorescence readings (Ex: λ_{485}/Em: λ_{520} nm) were taken every 5 min at 37 °C for 160 min in a microplate reader (TECAN GENios Plus). Final results were calculated based on the difference in the Area Under the fluorescence decay Curve (AUC) between the blank and each sample. The AUC formula was:

$$\text{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{20}/f_0$$

Results were expressed as micromolar TR equivalents (TE) calculated using the line equation from the standard curve:

$$\text{TE} = (Y - b)/m$$

where Y is the net AUC ($AUC_{sample} - AUC_{control}$), m is the slope and b is the Y-intercept.

2.5. Cell Culture

Highly invasive MDA-MB-231 human breast cancer cells (estrogen and progesterone receptor-negative), minimally invasive MCF7 human breast cancer cells (estrogen and progesterone receptor-positive) and immortalized non-tumorigenic MCF10A human breast epithelial cells, were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Breast tumor cells were grown as a monolayer culture in Minimum Essential Medium with Eagle's salts (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Hepes Buffer 1 M, 1% Sodium Pyruvate 100 mM and 1% Non-Essential Amino Acids mixture 100×. MCF10A cells were cultivated in HuMEC Ready Medium. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO_2. Cells were

routinely sub-cultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments.

2.6. Cell Proliferation Assay

Cell proliferation, measured as the cellular growth of treated cells *vs.* untreated controls, was measured using an XTT-based assay as described by Scudiero *et al.* [15] with some modifications. Briefly, cells were seeded at 2×10^3 cells/well (MCF7) or 1×10^3 cells/well (MDA-MB-231 and MCF10A) into 96-well culture plates (flat bottom) (100 μL of cell suspension/well). At 24 h after plating, 100 μL of fresh culture medium, with different concentrations of HT or TY was added in triplicate to the wells. Plates were incubated for 24 h or 24 h followed by a 48 h proliferation period with fresh medium at 37 °C and 5% CO_2. At these time points, medium was removed and 200 μL of fresh RPMI medium without phenol red that contained XTT (200 μg/mL) and PMS (20 μg/mL) was added. Plates were incubated for 3 h at 37 °C in 5% CO_2 and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

$$\text{viable cells (\%)} = (OD_{\text{treated cells}}/OD_{\text{control}}) \times 100$$

where OD is the difference in absorbance between optical density units ($OD = OD_{450} - OD_{620}$).

All measurements were performed in triplicate and each experiment was repeated at least three times.

2.7. Cell Cycle Assay

Cells were seeded in 12-well culture plates at 1×10^5 cells/well for MCF7 and MDA-MB-231 or at 5×10^4 cells/well for MCF10A for 48 h. Cells were then treated with different doses of HT or TY for 24 h. After incubation, cells were washed in cold PBS, fixed with cold 70% ethanol and stored at −20 °C for at least 24 h. At least 1×10^4 cells per sample were analyzed on an EPICS XL-MCL (Beckman Coulter, Spain) flow cytometer after propidium iodide labeling (PI/RNase Staining Buffer kit). The percentage of cells in G_0/G_1, S and G_2/M phases were calculated using FlowJo program (v5.7.2). Each experiment was repeated three independent times.

2.8. Apoptosis

The percentage of apoptosis was determined using a double staining assay with FITC-conjugated Annexin V and propidium iodide (PI). Briefly, after 24 h of cell exposure to the previously indicated doses of HT or TY in 12-well culture plates, cells were harvested, washed twice in cold PBS and resuspended in 100 μL 1× Annexin Binding Buffer. Cells were then stained with 5 μL Annexin V-FITC and 1 μL PI solution, gently vortexed, and incubated for 15 min at room temperature in the dark before flow cytometric analysis. As a positive control, cells were treated with 1 μM camptothecin (CPT). Each experiment was repeated three independent times.

2.9. Reactive Oxygen Species Detection

Intracellular reactive oxygen species (ROS) level was measured using a cell-permeable fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as we described previously [16]. In brief, cells were seeded into 96-well culture plates at 1×10^4 cells/well (MCF7, MDA-MB-231 cells) or 5.5×10^3 cells/well (MCF-10A cells). After 24 h at 37 °C and 5% CO_2, cells were treated with different doses of HT or TY for 24 h. Cells were then washed twice with Hank's Buffered Salt Solution (HBSS) and incubated with fresh DCFH-DA (100 μM) in HBSS for 30 min at 37 °C in 5% CO_2. DCFH-DA stock solution (20.5 mM) was prepared in DMSO and stored at −20 °C for maximum one month. After that, cells were washed twice in HBSS, and wells were filled with 100 μL HBSS before fluorescence acquisition in a plate reader (TECAN GENios Plus) (Ex: λ_{485}/Em: λ_{535} nm, Gain 60). Intracellular ROS level percentage was calculated as follows:

$$F = [(F_{t_{30}} - F_{t_0})/F_{t_0}] \times 100$$

where F_{t_0} is the fluorescence at $t = 0$ min and $F_{t_{30}}$ the fluorescence at $t = 30$ min.

It has been reported that the addition of H_2O_2 increases oxidative stress in cultured cells [17]. Therefore, in order to evaluate the protective capacity of HT or TY against induced oxidative stress, H_2O_2 (500 μM) was added to the wells after removal of assay medium. This allows avoiding a direct reaction in the medium between these compounds and the oxidant source. After 30 min at 37 °C, fluorescence was quantified as described above.

All tests were run in triplicate for each experimental condition and each experiment was repeated at least three times. All experiments were conducted using iron-free media (MEM and HuMEC).

2.10. Alkaline Single-Cell Gel Electrophoresis (Comet Assay)

At 24 h, cells treated with HT or TY were scraped into 12-well culture plates, washed twice (300× *g* 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free) and resuspended in 1 mL of cold 1× PBS. In order to evaluate the ability of HT and TY to prevent oxidative DNA damage, cell suspensions were exposed for 10 min to 50 μM H_2O_2 at 4 °C. After that, cells were washed twice and frozen in FBS-DMSO (90:10, v/v) at −80 °C until the Comet assay procedure.

DNA single strand break by alkaline microgel electrophoresis was performed according to Singh *et al*. [18] with some modifications. Cells were thawed in a bath at 37 °C, centrifuged (300× *g* 10 min, 4 °C) in cold MEM with 25% FBS and resuspended in cold 1× PBS to a density of 1.65×10^5 cells/mL. Cells were then suspended in melted and cooled (at 40 °C) low melting point agarose (LMA). Cell suspensions (50 μL) were spread over a sample area of pre-warmed 1% normal melting point agarose (NMA) precoated CometSlide™ slides. After 15 min at 4 °C in the dark, slides were immersed in cold Lysis Solution (Trevigen, Inc.) at 4 °C for 30 min to dissolve lipids and proteins. In order to separate the two DNA strands, slides were then immersed in fresh Alkaline Solution (pH > 13) for 30 min at room temperature in the dark. Electrophoresis was performed in an Ebony acrylic electrophoresis tank with a cooled platform containing cold Alkaline Electrophoresis Solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 25V (1 V/cm) and 300 mA for 40 min. The slides were washed twice with distilled water for 10 min and neutralized with 10 mM Tris-HCl, pH 7.5 for 5 min,

followed by immersion in 70% ethanol for 5 min and air-dried overnight at room temperature. Slides were stained with Sybr® green before scoring.

2.11. Slide Scoring and Analysis

DNA strand breaks were examined using a fluorescence microscope (Zeiss Axiovert 200) equipped with a Luca EMCCD camera (Andor Technology, Belfast, UK) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). Fifty cell images were randomly characterized per sample using 20× magnification. Relative fluorescence between head and tail through the olive tail moment (Olive_TM) was used to determine DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

$$\text{Olive\_TM} = [(\text{Tail (mean)} - \text{Head (mean)}) \times \text{Tail (\% DNA)}]/100$$

2.12. Statistical Analysis

Results are presented as mean (±SEM), except for cell proliferation results. For this assay, results are presented as mean (±SD). Results are expressed as a percentage relative to the control, which was defined as 100%. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Values of $p < 0.05$ were considered significant. Statgraphics Plus 5.1 statistical software (Statpoint Technologies, Inc., Warrenton, VA, USA) was used for the statistical analysis.

3. Results

3.1. Effect of HT and TY on Radical Scavenging Activity

Radical scavenging capacity was determined using DPPH, ABTS and ORAC assays.

The antiradical activity of HT and TY, measured by scavenging activity in the DPPH radical assay, indicated that HT at up to 10 mole ratio (mole antioxidant/mole DPPH) exerts a slightly higher scavenging activity than TOC while TY does not possess a radical scavenger activity (Table 1(a)).

The ABTS antiradical assay showed that HT was more effective than TR in scavenging the ABTS cationic radical while TY exhibited a maximum 85% RSA at 800 μM (Table 1(b)).

The peroxyl radical scavenging activity of HT and TY, measured by the $ORAC_{FL}$ assay, showed a protective effect against AAPH-induced peroxyl radical activity for both phenols. Both exerted higher protection against the peroxyl radical than TR for low concentrations up to 100 μM (Table 1(c)).

3.2. Cell Proliferation

To investigate the effect of HT and TY on human breast cell growth, cells were treated with concentrations of HT or TY ranging from 1 to 100 μM for 24 h. Neither HT nor TY had significant effects on the cell proliferation rates of MCF7, MDA-MB-231 and MCF10A cells (Figure 1(a)), even after an additional 48 h with fresh medium (Figure 1(b)). We also investigated the potential antiproliferative effect of these compounds at high, non-physiological concentrations up to 1000 or

5000 μM of HT or TY, respectively. HT showed a dose-dependent reduction of cell proliferation in the three cell lines from a concentration of 200 μM with an absence of viability observed at 1000 μM, while TY did not affect cell viability at any concentration assayed (data not shown).

No marked changes in cell morphology were observed by light microscopy in any of the cell lines tested when concentrations between 1 and 100 μM of HT or TY were used (data not shown).

Table 1. Antioxidant activity of hydroxytyrosol (HT) or tyrosol (TY) quantified as Radical Scavenging Activity (RSA) by (**a**) DPPH assay (% RSA at 50 min) and (**b**) ABTS assay (% RSA at 30 min); (**c**) Antioxidant activity quantified as Trolox Equivalent (TE) by $ORAC_{FL}$ assay. Trolox™ (TR) and α-tocopherol (TOC) were used as antioxidant references.

(**a**)

mole AH/ mole DPPH	HT	TY	TOC
0	3.96	3.64	3.63
0.06	36.24	n.d.	23.21
0.13	71.47	5.20	48.16
0.25	96.40	4.25	75.85
0.5	97.80	3.02	90.17
1	98.07	2.59	95.98
2.5	n.d.	2.34	n.d.
5	n.d.	3.44	n.d.
10	n.d.	3.37	n.d.

n.d.: not determined.

(**b**)

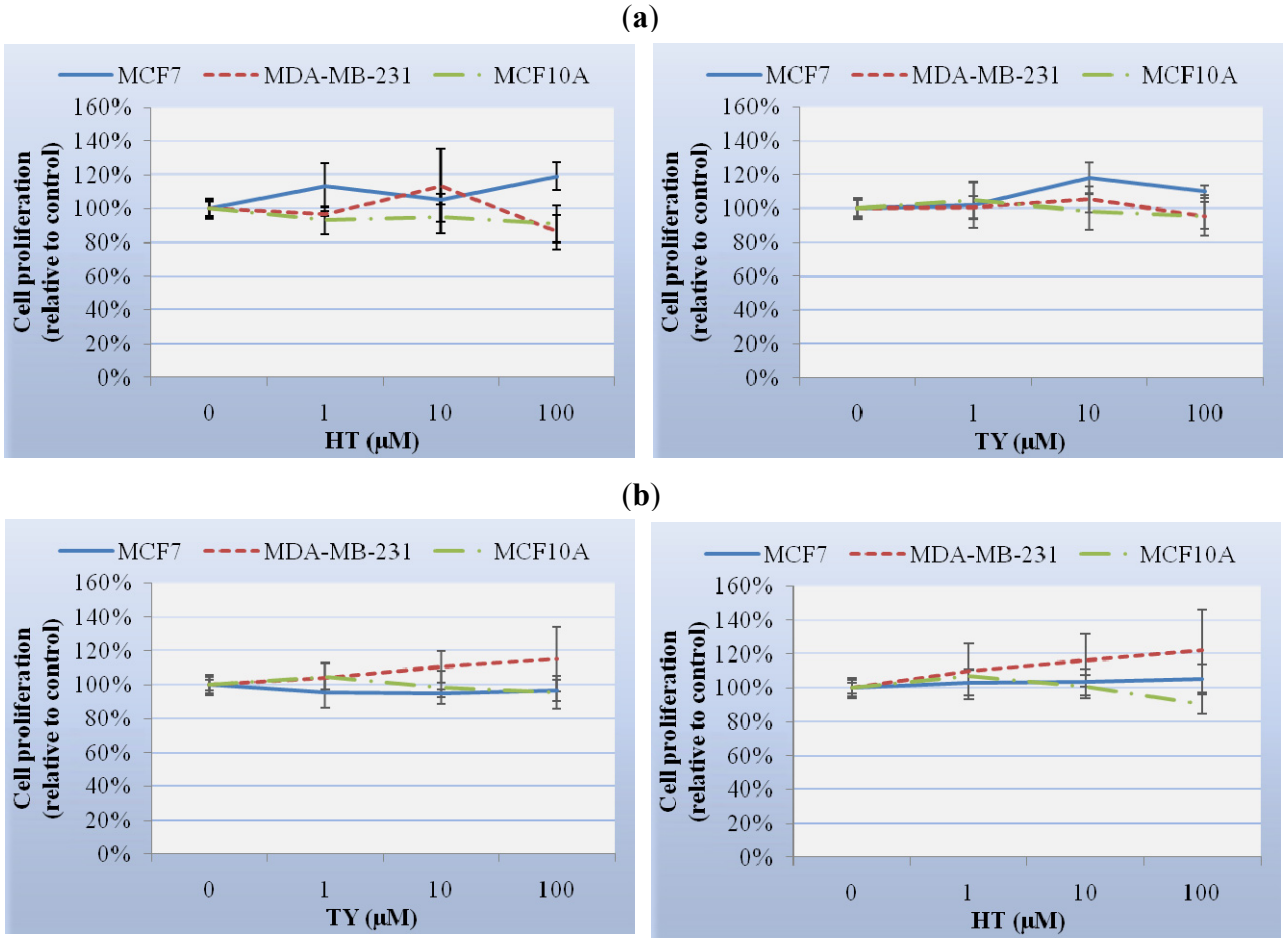
μM	HT	TY	TR
6	5.07	10.31	n.d.
12.5	5.45	14.32	n.d.
25	8.42	20.14	n.d.
50	18.09	31.81	16.21
100	36.63	44.55	29.39
200	69.53	58.25	50.07
400	96.30	73.82	88.03
800	99.47	85.04	99.48

n.d.: not determined.

(**c**)

TE (μM)	HT (μM)	TY (μM)
3.12	14.82	1.64
6.25	30.97	8.30
12.5	50.37	20.84
25	92.24	58.35
50	150.43	106.76
100	262.76	205.57

Figure 1. Cell proliferation assay measured with XTT tetrazolium salt (**a**) after 24 h of HT or TY exposure, or (**b**) after 24 h of HT or TY exposure followed by 48 h with fresh medium. Data are the mean (±SD) relative to an untreated control of three independent assays carried out in triplicate.



3.3. Cell Cycle and Apoptosis

To evaluate whether HT or TY interfered with the cell cycle or the induction of apoptosis, MCF7, MDA-MB-231 and MCF10A cells were treated for 24 h with increasing concentrations of HT or TY (between 10 and 200 µM). The results revealed that HT and TY did not alter the cell cycle in any of the cell lines studied (data not shown).

Flow cytometric analysis of apoptosis revealed that treatment with HT or TY for 24 h did not induce apoptosis in MCF10A cells or in MCF7 or MDA-MB-231 cells when compared to the controls (data not shown).

3.4. Intracellular ROS Level

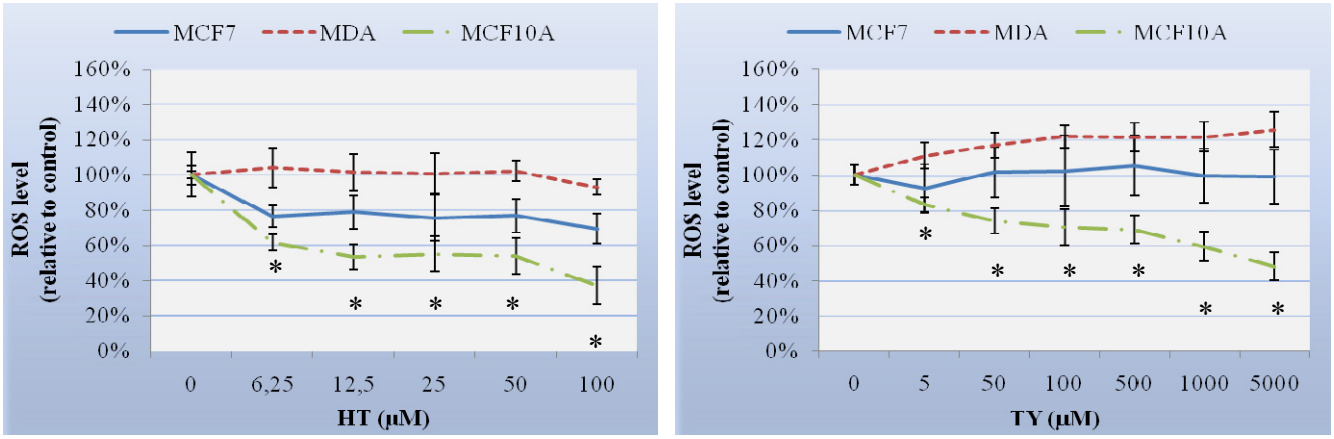
Intracellular reactive oxygen species (ROS) were quantified by the dichlorofluorescein diacetate (DCFH-DA) assay using a microplate reader. Results showed a dose-dependent decrease in ROS level of MCF10A cells treated for 24 h with either HT or TY. However, HT and TY failed to significantly decrease intracellular ROS level in either MCF7 or MDA-MB-231 cells (Figure 2(a)). While HT

reduced ROS level by up to 20% in MCF7 cells, this reduction was not considered statistically significant ($p = 0.34$).

H_2O_2 effectively induced oxidative stress in both, human breast cancer cells and human breast epithelial cells (Figure 2(b)). In order to investigate the *in vitro* preventive effect of HT or TY against H_2O_2-mediated oxidative stress, we measured the intracellular ROS level in cells treated with HT or TY for 24 h. As can be seen in Figure 2(c), MCF10A cells treated with HT or TY showed a significant dose-dependent decrease in ROS production compared to the control. In addition, HT was also able to decrease the ROS level in MCF7 and MDA-MB-231 cells induced by H_2O_2 exposure. It is worth mentioning that the decrease in ROS level was greater in the breast epithelial cell line than in the breast cancer cell lines. On the other hand, TY did not decrease ROS level in MCF7 or MDA-MB-231 cells at concentrations up to 5000 μM (Figure 2(c)).

Figure 2. (**a**) Intracellular reactive oxygen species (ROS) in breast cells treated for 24 h with HT or TY; (**b**) Increase of the cellular ROS level after an oxidative burn with H_2O_2; (**c**) Intracellular ROS in breast cells treated for 24 h with HT or TY followed by an oxidative burst with H_2O_2. Inhibitory effects of HT and TY are shown as percent inhibition of untreated or H_2O_2-stimulated fluorescence and represented as the mean ± SEM of three independent replicates carried out in triplicate. † MCF7; ‡ MDA-MB-231; * MCF10A indicates significant differences.

(a)



(b)

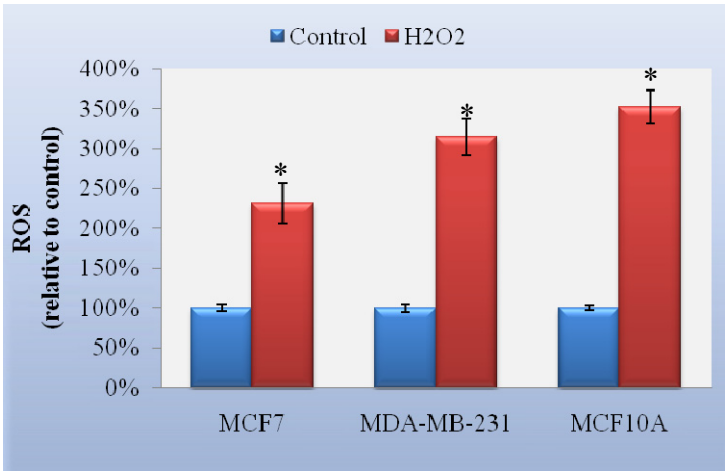
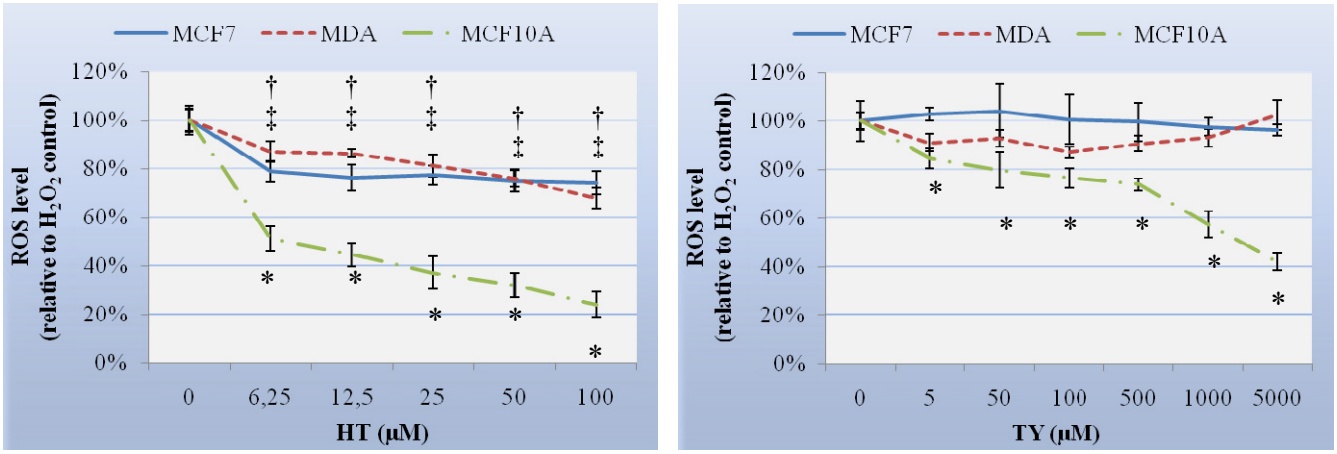


Figure 2. *Cont.*

(c)



IC_{20} and IC_{50} values were defined as the values for 20% and 50% antioxidant inhibition of basal or H_2O_2-stimulated fluorescence in DCFH-DA probes. The Relative Antioxidant Value (RAV) ratio was found to be a good parameter for the determination of oxidative inhibition profiles.

$$RAV = [(IC_{20\,(PH)}/IC_{20\,(TOC)}) + (IC_{50\,(PH)}/IC_{50\,(TOC)})]/2$$

where PH is the compound (simple phenol) and TOC is the reference (α-tocopherol).

Our results showed that TY has a RAV about 46-fold higher than TOC for MCF10A cells whereas HT only has 1.44-fold higher. This indicated much high antioxidant activity of HT compared with TY in normal breast cells, but less than TOC (Table 2). In MCF7 and MDA-MB-231 cells, a 50% antioxidant inhibition was not observed; therefore, RAV ratios were not determined in these cell lines.

Interestingly, in H_2O_2-stimulated MCF10A cells, the RAV ratio of TY was 42-fold higher than TOC, indicating a very low antioxidant capacity in normal breast cells. The 0.67-fold difference between RAV ratio of HT and TOC is of particular interest, due to the high antioxidant activity of HT in H_2O_2-stimulated MCF10A cells (Table 2).

Table 2. Oxidative inhibition in MCF10A cells. IC_{20} and IC_{50} values defined as the values for antioxidant inhibition of basal or H_2O_2-stimulated fluorescence in DCFH-DA assays and the Relative Antioxidant Value (RAV) as a parameter for the relative determination of oxidative inhibition profiles compared to α-tocopherol.

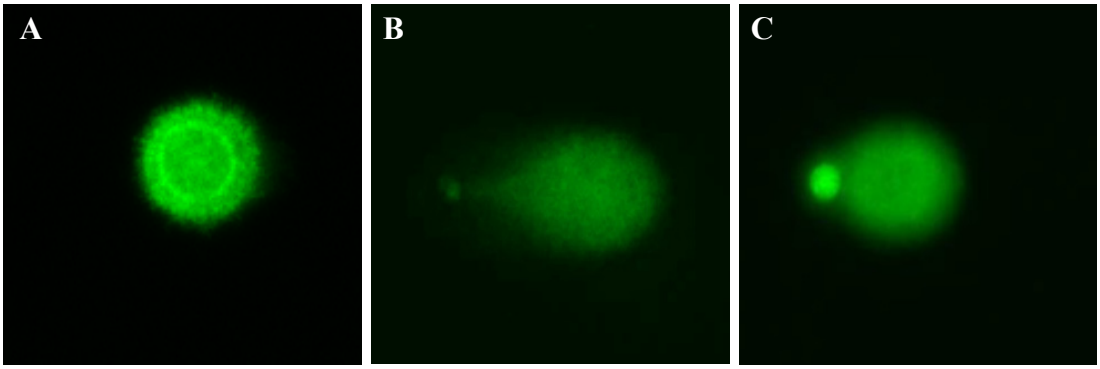
	Basal			**H_2O_2-stimulated**		
	HT (µM)	**TY (µM)**	**TOC (µM)**	**HT (µM)**	**TY (µM)**	**TOC (µM)**
IC_{20}	3.52	4.04	4.33	2.66	65.36	2.49
IC_{50}	65.64	2942.60	31.89	20.66	4244.40	73.50
RAV	1.44	46.60	1.00	0.67	42.00	1.00

3.5. Effect of HT and TY on Oxidative DNA Damage

The ability of H_2O_2 to induce DNA strand breaks in these human breast epithelial cell lines was examined using the Comet assay. In untreated cells, DNA does not migrate far from the origin when examined by alkaline microgel electrophoresis (Figure 3(a)). Following H_2O_2 exposure, control and

pretreated breast cells with damaged DNA have the shape of a comet, the tail length and fluorescent intensity of which are related to the number of DNA strand breaks induced by the DNA-damaging agent (Figure 3(b,c)).

Figure 3. Representative images of Comet assay analysis of MCF10A cells. (**a**) Untreated cell, showing a circular shape indicating absence of DNA damage; (**b**) 10 min. H_2O_2 exposed cell, exhibiting a long and bright tail related to DNA strand breaks, indicating DNA oxidative damage; (**c**) 10 min. H_2O_2 exposed cell after 24 h of 100 µM HT pretreatment, illustrating the reduction of tail length and fluorescent intensity indicative of reduced DNA damage.



Breast cells exposed to H_2O_2 were effectively DNA damaged and the mean olive tail moment (Olive_TM) was determined by the Comet assay. Breast epithelial cells were the most sensitive to the H_2O_2-induced DNA damage (Figure 4(a)).

In unexposed cells, HT reduced DNA damage significantly in MCF7, MDA-MB-231 and MCF10A cells whereas TY only reduced it in MCF10A cells (Figure 4(b)). In H_2O_2-exposed cells, HT showed a preventive DNA damage effect in the three cell lines whereas TY was unable to reduce Olive_TM in any of the cell lines; indeed, in MDA-MB-231 cells, TY increased Olive_TM significantly.

Figure 4. Olive Tail Moment (Olive_TM) as the mean ± SEM for three independent assays. (**a**) After an H_2O_2 injury; (**b**) after 24 h of HT or TY treatment, and (**c**) after 24 h of HT or TY treatment followed by an H_2O_2 injury.

(a)

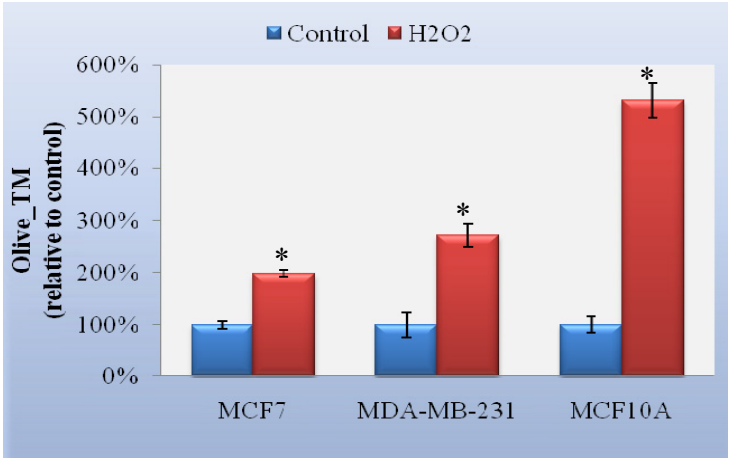
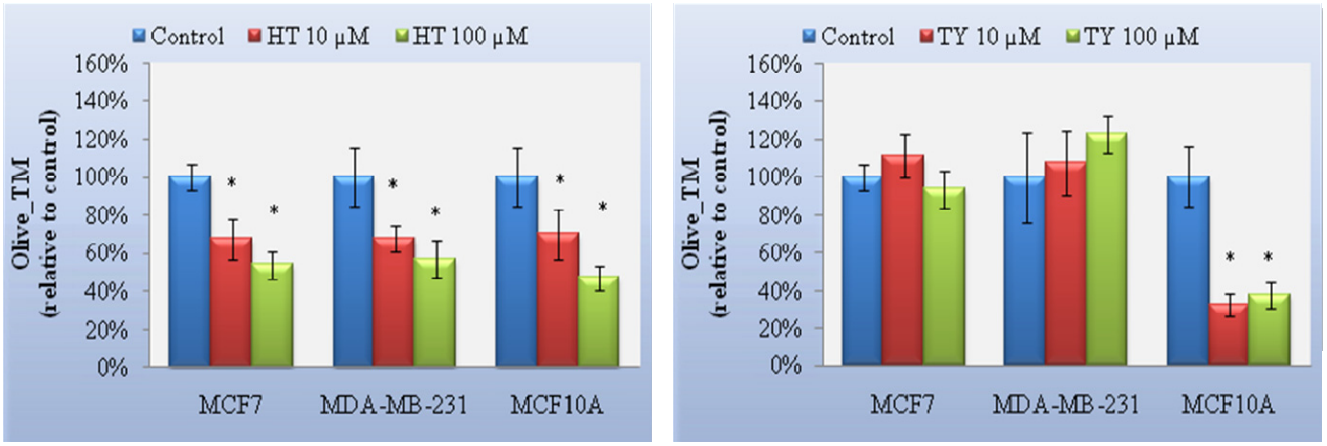
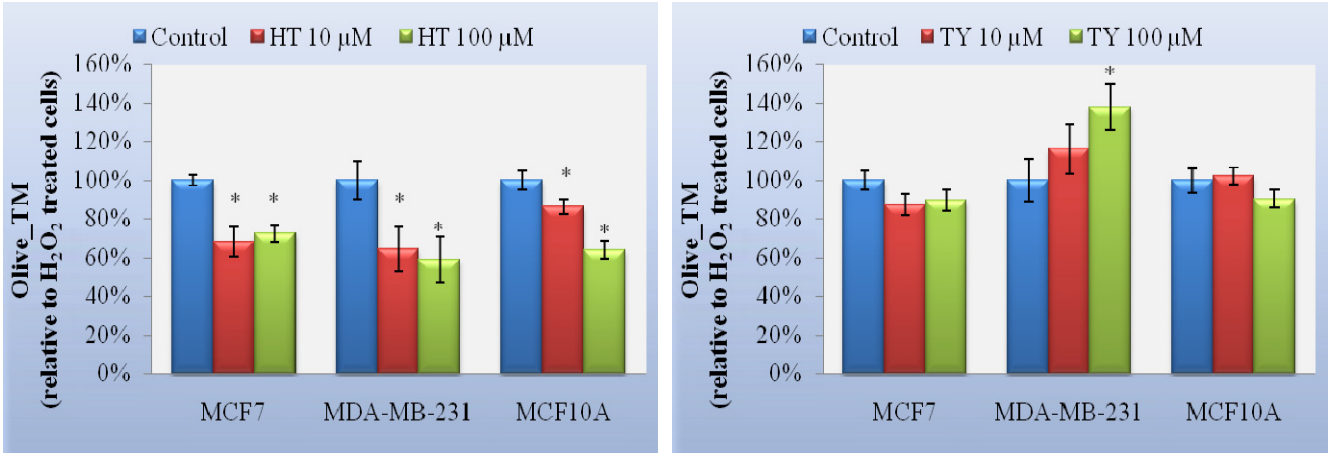


Figure 4. *Cont.*

(b)



(c)



4. Discussion

There is some scientific evidence relating Mediterranean dietary pattern with a lower incidence of cardiovascular diseases and cancer, among other diseases. Virgin olive oils (VOOs) represent the main source of fats in this diet and it has been demonstrated that consumption of VOOs reduces human arterial hypertension, lipid peroxidation of membranes, tumor incidence and number of tumors [19,20]. Minor compounds play a key role in VOOs' healthy properties. Among them, phenols have demonstrated healthy bioactivity properties. Interest in phenolic compounds has increased greatly, with attention being focused on finding naturally occurring antioxidants for foods or medical uses to replace synthetic antioxidants that, in some cases, have been reported to be carcinogenic [21].

HT and TY are two of the major simple phenols present in VOOs as simple form or conjugates [2]. Bioavailability studies have demonstrated that they are dose-dependently absorbed in animals and humans after olive oil ingestion [22], accumulated in the body and, finally, systemically exert biological effects [23].

The present work describes the antioxidant capacity of HT and TY molecules using chemical and cellular assays and their relationship with proliferation of human breast tumor *vs.* normal cells.

HT and TY are structurally identical except that HT has an extra –OH group forming a catechol group, which is considered responsible for its higher antioxidant activity. This catechol group is able to stabilize free radicals through the formation of intermolecular hydrogen bonds [8]. In our chemical analysis, the catechol phenol HT exhibited a strong antioxidant activity in DPPH, ABTS and ORAC assays, while TY, without a catechol group, showed a weak antioxidant activity in DPPH assay. Remarkably, TY acts as an efficient scavenger against ABTS and AAPH radicals, although to a lesser extent than HT, indicating the minor importance of the cathecol group in cationic or peroxylic radicals' scavenging activities. These results are in agreement with those previously reported by Visioli *et al*. [8] affirming that HT and, to a lesser degree, TY are more potent scavengers of free radicals than vitamin E.

Although nowadays there is no scientific evidence relating to the physiological concentrations of HT or TY after olive oil ingestion, some authors have suggested it could be between 10 and 100 μM [24]. Cell treatment with HT or TY in the range of their possible physiological concentrations (1–100 μM) did not have any effect on cell proliferation in any of the cell lines studied, independently of the exposure times. However, HT dramatically reduced the viability of MCF7, MDA-MB-231 and MCF10A cell lines when used at concentrations from 200 μM to 1000 μM. Fabiani *et al*. described such an effect in colon adenocarcinoma HT29 cells [25]. Furthermore, HT and TY did not alter the cell cycle or induce apoptosis in these cell lines. Although these results are in agreement with those achieved in LLC-PK1 renal cells, they are in contrast with results in human promyelocytic leukaemia HL60 cells with a noticeable antiproliferative, cell cycle arrest and apoptotic effect of HT. Otherwise, TY showed no antiproliferative effect in HL60 cells [7,25].

HT or TY's inability to inhibit breast cancer cell proliferation at the assayed times and concentrations, suggests that they cannot protect against breast cancer once developed. Quiles *et al*. [24] described the lack of inhibition of HT or TY in PC3 cells treated with 10 to 250 μM, as did Menendez *et al*. [26] in SKBR3 and MCF7 cells after 5 days of HT or TY treatments in the range of 6.25 to 100 μM. Moreover, Sirianni *et al*. [27] recently described the dose-dependent inhibition of MCF7 cell proliferation by HT and oleuropein (OL) with treatments of 1 to 100 μM; cell growth was induced by 17-β-estradiol (E_2). In addition, HT and OL are not able to interfere with estrogen action through competition with estrogen receptors (ER), which are responsible for activation of the gene expression involved in cell proliferation.

In order to clarify how nutritional antioxidants are able to prevent or treat oxidative damage, Berger [28] affirmed that nutrients cannot treat an installed disease, such as gastrointestinal cancer, but that they may prevent its promotion. Indeed, the answer to the question: "Can installed damage caused by ROS be treated by antioxidant nutrients?" is "probably not", but the answer to the question: "Can oxidative damage be treated nutritionally?" is "yes" [28].

Growing evidence supports the hypothesis that risk factors such as lifestyle, age, environment, diet, drinking, smoke, *etc.* are determinants in breast neoplastic transformation, and are closely associated with a chronic increase in the basal level of oxidative stress. A decrease in oxidative stress state could prevent the development of tumors and, potentially, cancer. In fact, serum markers for oxidative DNA damage have been shown to increase in women diagnosed with breast cancer [29]. On the other hand, it has been suggested that consumption of VOOs, which are particularly rich in phenolic antioxidants, such as HT and TY, should afford considerable protection against breast cancer by inhibiting oxidative

stress [2]. In our study we demonstrated that HT and TY reduce basal and H_2O_2-induced ROS level in breast epithelial MCF10A cells, whereas TY failed to reduce both in MCF7 or MDA-MB-231 cells and HT only reduced H_2O_2-induced ROS level slightly in breast cancer cells. These results point to a differential antioxidant activity of both compounds between normal breast and tumor cells as we described for squalene [16]. Thus, we suggest that HT and TY could prevent oxidative stress in normal breast cells, thereby preventing the initiation of a chain of reactions to transform normal cells into cancer cells. Noticeably, it is necessary to use a much larger amount of TY to obtain the same ROS reduction level as HT in MCF10A cells (Table 2). Up to 100 μM concentrations of HT and TY used in the present study are probably within the physiological range. However, 500 to 5000 μM of TY exceed this range and could be regarded as being in the pharmacological range.

Di Bendeto *et al.* [11] described differences between HT and TY in inhibiting cell-mediated oxidation of LDL (100% HT *vs.* 40% TY) in J774 A.1 macrophage cells due to its intracellular presence. Thus, time-dependent TY, accumulated inside the cell was effective only at later time-points (24 h) or at higher concentrations than HT, which was rapidly found inside the cells and disappeared within 18 h. Thus, we can presume a quick antioxidant defense by HT followed by a slower defense by TY upon VOO intake.

Estrogens, known human breast pro-carcinogens, exert their actions by two mechanisms; the ER-dependent mechanism, involving the activation of ER and subsequent stimulation of cell growth and proliferation [30] or the ER-independent mechanism, involving the generation of genotoxic estrogen metabolites, which are highly reactive and damage DNA by the formation of free radicals and consequently ROS [30]. In accordance with Sirianni *et al.* [27], HT inhibition of E_2-induced MCF7 proliferation does not involve the ER-dependent mechanism but points to an inhibition of the E_2 signaling pathway. Felty *et al.* [31] identified mitochondria as a major source of E_2-induced ROS (mtROS) in breast cancer cells and described mtROS as a messenger involved in signaling pathways of cell proliferation control, increasing the transcription of cell cycle genes. These authors found the same amount of mtROS in ER-negative MDA-MB-468 cells and in ER-positive MCF7 or T47D cells, suggesting that mtROS production does not depend on the presence of ER in breast cancer cells. If mtROS acts as a messenger in breast cancer proliferation, it could explain why an antioxidant such as HT reduces E_2-induced cell proliferation, as described by Sirianni *et al.*, whereas in the same concentrations without E_2 stimulation we do not detect any significant growth alteration.

Cellular protection against oxidative stress is provided by two types of antioxidants; direct antioxidants with a redox activity; and indirect antioxidants (redox active or not) which activated the Nrf2/ARE pathway resulting in transcription of phase II enzymes such as glutathione S-transferase, NAD(P)H: quinone oxidoreductase 1 or glutathione reductase [32]. In addition to the fact that HT and TY act as direct antioxidants, they could also be indirect antioxidants activating the nuclear factor-like 2 (Nrf2). Nrf2, considered a key factor in the cellular defense mechanisms against oxidative stress, might be induced more strongly in MCF10A cells than in MCF7 and might have little or no effect in MDA-MB-231 cells, explaining the differential protection effect of HT and TY on intracellular ROS level. To the best of our knowledge, until now, only Liu *et al*. [33] have described the protection of HT on ARPE-19 human retinal pigment epithelial cell line from oxidative stress induced by acrolein, a major component of cigarette smoke. Further studies will be necessary to

elucidate the possible Nrf2/ARE pathway intervention of HT and TY differential antioxidant activity in breast cell lines.

HT has been described as preventing DNA damage beyond its antioxidant capacity, as it can affect a range of enzymes, including cyclooxygenase and NAD(P)H oxidase while TY has no protective effect [8]. In accordance with these authors, our findings point to a protective effect of HT against basal and H_2O_2-induced DNA damage regardless of the breast cellular type, whereas TY only has a protective effect on ROS basal level in non-tumoral breast cells.

Both compounds reduce intracellular ROS level and oxidative DNA damage in normal breast cells. This could protect against cellular mutations, preventing carcinogenesis. However, when the disease has occurred, oxidative status in the malignancy place is altered. In this condition, while HT still protects non-tumor breast cells against DNA damage, TY fails to protect them at physiological concentrations. Although HT contributes to reduce DNA damage in normal breast cells, it protects breast tumor cells too. Accordingly, our results must be interpreted carefully, because a reduction of DNA damage in cancer cells might promote cell growth and might inhibit the action of anthracycline chemotherapeutic agents, such as doxorubicin, which induces apoptosis of cancer cells by the oxidative damage resulting from enhanced oxidative state of the cells or, in contrast, might reduce ROS messenger signaling of proliferation resulting in a reduction of tumor cell growth. In any case, we have not detected any modulation of the growth activity *in vitro* in breast cancer cells after HT or TY treatment at the assayed times.

In this paper, HT has been described as an antioxidant compound with higher activity than TY and related to the prevention of breast cancer, but we must not forget that VOO´s minor compounds can interact with each other, potentiating or inhibiting the effects described for each component alone. Our results indicate some healthy properties of these two simple phenols which may be of interest in pharmacology or as a nutritional supplement or could even lead to establishing the ideal concentrations of each component in VOOs in order to label it as a healthy oil. However, we must be prudent about extrapolating these results regarding epidemiological olive oil health impacts. Future work is needed to investigate these synergetic or inhibitory effects.

5. Conclusions

The simple phenol HT could contribute to the preventive cancer activity attributed to VOOs due to the reduction of oxidative stress and oxidative DNA protection in normal breast cells at physiological concentrations, whereas TY is needed at pharmacological concentrations to reduce oxidative stress and fails to protect DNA damage against an oxidative burst.

Both phenols exert a selective antioxidant defense, preventing oxidation in normal breast cells but not in breast cancer cells, which could be helpful to cancer therapies that increase oxidative stress. HT also prevents induced DNA damage in cancer cells, so it might interfere with these therapies.

Although *in vitro* studies have pointed to a preventive role of HT against human breast cancer, the precise mechanisms of action remain to be clarified. Further studies are necessary to elucidate the cellular signaling events that HT and TY target in oxidative stress protection and subsequent breast cancer prevention.

Acknowledgments

This study was supported by the "Instituto Andaluz de Biotecnología" (BIOÁNDALUS 08/22/L5.3); Ministerio de Ciencia e Innovación-FEDER (RTA2008-00066-C03-03); and "Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud" (CEAS).

Conflict of Interest

The authors declare no conflict of interest.

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Molecular Aspects of Squalene and Implications for Olive Oil and the Mediterranean Diet

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- Capítulo del libro: *"The Mediterranean Diet: An Evidence-Based Approach"*
- Fecha de publicación: Octubre 2014
- Páginas: 281-290
- Idioma: Inglés
- DOI: 10.1016/B978-0-12-407849-9.00026-9
- ISBN: 978-0-12-407849-9

The Mediterranean Diet

An Evidence-Based Approach

EDITED BY

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Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN : 978-0-12-407849-9

Typeset by Spi

Printed and bound in United States of America
15 16 17 18 19 10 9 8 7 6 5 4 3 2 1

Chapter 26

Molecular Aspects of Squalene and Implications for Olive Oil and the Mediterranean Diet

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ABBREVIATIONS

DNA	deoxyribonucleic acid
HDL	high-density lipoprotein
LDL	low-density lipoprotein
ROS	reactive oxygen species
SSL	skin surface lipids
UV light	ultraviolet light
VLDL	very low-density lipoprotein

MEDITERRANEAN DIET

The effect of diet on human health has been widely reported in many scientific studies, providing evidence that a dietary pattern rich in some beneficial food groups can reduce the incidence of various chronic degenerative diseases [1]. In this sense, the Mediterranean diet is considered an example of healthy diet. Several public health-based studies have revealed that adherence to the Mediterranean diet is associated with longevity and a lower incidence of major chronic degenerative diseases such as cardiovascular diseases and certain cancers [2]. Unfortunately, changes in diets and lifestyles have been accelerated over the past three decades in developing and in-transition countries. Over the past 20 years there has been a generalized decrease in adherence to the Mediterranean diet in southern European countries. This is having a significant effect on the health and nutritional status of these populations. Therefore, it is important to promote a healthy diet, especially among young people.

VIRGIN OLIVE OIL

Although there are variations in the components of the traditional Mediterranean diet between countries, olive oil represents a distinctive element, being the main source of dietary lipids. Thus, olive oil is considered a key component of the Mediterranean diet. It has been described that virgin olive oil is effective in preventing and/or reducing hypercholesterolemia, serum lipoprotein concentrations, atherosclerosis, hypertension, cardiovascular diseases and thrombotic risk, oxidation and oxidative stress, obesity, type 2 diabetes, inflammatory processes, and cancer [3]. Recently, Estruch et al. [4] conducted a primary prevention trial and observed that an energy-unrestricted Mediterranean diet supplemented with extra virgin olive oil resulted in a substantial reduction in the risk of major cardiovascular events among high-risk participants.

Virgin olive oil is obtained from the fruit of the olive tree (*Olea europaea sativa*) by mechanical or other physical means under conditions, particularly thermal, that do not lead to alteration of the oil. Both virgin and extra virgin olive oil are produced by simply crushing olives and extracting the juice in a press or centrifuge. Neither virgin and extra virgin olive oil are subjected to any treatment except washing, decantation, centrifugation, and filtration. This process retains most of the compounds originally present in the olive fruit, which are responsible for the characteristic taste, flavor, and color of the

oil and which contribute to the beneficial health effects of virgin olive oil. Consequently, virgin olive oil is a natural olive juice; it is the only vegetal fat that is obtained from the fruit, whereas the rest are obtained from seeds. Indeed, other edible oils such as sunflower, soybean, and rapeseed canola oils must be refined before consumption, and their original composition changes during this process.

The chemical composition of olive oils may vary depending on different factors including olive variety, the ripening stage of the fruit at the time of collection, and the method used to process the olive fruit. Overall, the chemical composition of virgin olive oil consists of major and minor components. Major components represent about 98–99% of the total oil weight and are composed mainly of triacylglycerols. Oleic acid (a monounsaturated fatty acid) is present in a much higher concentration (55–83%) than the other acids (linoleic, palmitic, or stearic acids). Minor components, present in small amounts (about 2% of oil weight), include more than 230 chemical compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants. Virgin olive oil is particularly appreciated by consumers for its high content of healthy compounds such as oleic acid and numerous functional bioactive components. In addition, the latter also contribute to the unique flavor and taste of virgin olive oil.

In the same way, both the US Food and Drug Administration and the European Food Safety Authority confirmed the health benefits of some of the components present in virgin olive oil. In 2004, the US Food and Drug Administration allowed a claim on olive oil labels concerning "the benefits on the risk of coronary heart disease of eating about two tablespoons (23 g) of olive oil daily due to the monounsaturated fat in olive oil." Moreover, in 2012, the *Official Journal of the European Union* published a list of health claims that may be made on foods (Commission Regulation [EU] No. 432/2012). Among them are two health claims directly related to olive oil. Oleic acid is the subject of one of the health claims, which is as follows: "Replacing saturated fats in the diet with unsaturated fats contributes to the maintenance of normal blood cholesterol levels. Oleic acid is an unsaturated fat." The second claim involves the olive oil polyphenols and their related health benefits, as follows: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress." However, there is growing interest in studying the biological effects of an increasing number of minor components present in virgin olive oils. In recent years, there much scientific evidence has been published by researchers all over the world. Based on this evidence, olive oil can be categorized as a functional food that contains, in addition to a high level of oleic acid, other medicinally important minor components with multiple biological activities.

SQUALENE IN VIRGIN OLIVE OIL

One interesting compound is squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane), which is a terpenoid hydrocarbon present at high concentrations in virgin olive oils. Thus squalene is a polyunsaturated hydrocarbon with the formula $C_{30}H_{50}$. It is the major hydrocarbon present in virgin olive oil (>90%), with content ranging from 0.8 to 13 g/kg [5]. Olive oil contains up to 300-fold more squalene than other vegetable oils and up to 5000-fold more than some vegetable foods [6]. Squalene content in olive oil depends on the olive cultivar and oil extraction technology, and it is considerably reduced during the process of refining. It is important to note that extra virgin olive oil contains significantly higher concentrations of squalene than refined olive oil and seed oils.

In addition to the health benefits, virgin olive oil exhibits high resistance against oxidation compared with other vegetable oils. This is an important feature because the physicochemical changes associated with autoxidation result in the loss of sensory and nutritional values. Indeed, it has been suggested that squalene makes a limited contribution to the oxidative stability of virgin olive oil at ambient or slightly elevated temperatures, but it seems to play a more significant role in the protection of the quality of olive oil during exposure to frying temperatures [7].

All plants and animals, including humans, are capable of producing squalene. Plant sterols, also called phytosterols, are biosynthetically derived from squalene (Figure 1). In olive oil, sterols are present in the range of 1800–4939 mg/kg. These compounds are known to reduce serum concentrations of low-density lipoprotein (LDL) cholesterol in both humans and animals [8].

SQUALENE IN HUMANS

Squalene in mammalian cells originates partly from endogenous cholesterol synthesis and partly from dietary sources, especially in populations consuming large amounts of olive oil. In humans, squalene is synthesized in the liver and the skin, transported in the blood by very low-density lipoproteins (VLDL) and LDLs, and secreted in large quantities by the sebaceous glands [9]. The greatest concentration of squalene occurs in the skin; it is one of the major components of skin surface lipids (SSLs) (Table 1).

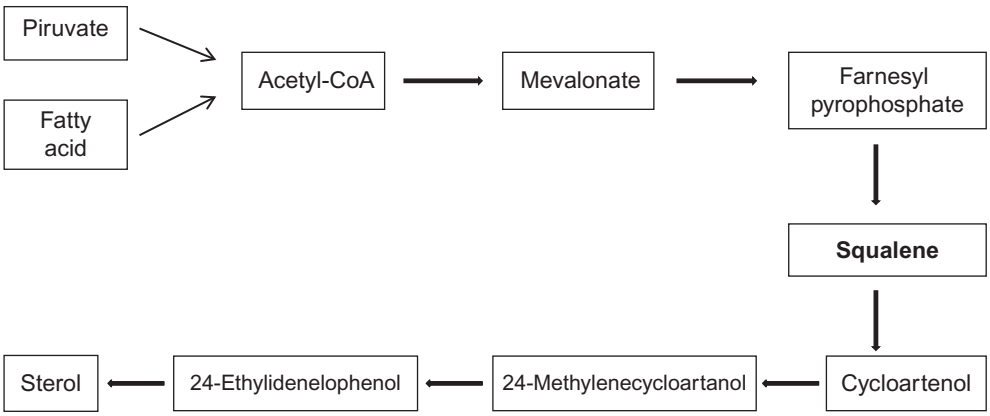

FIGURE 1 Sterols synthesis pathway in olive fruit.

TABLE 1 Composition of Sebum in Humans

Substance	Composition (%)
Vax esters	25
Squalene	13
Cholesterol	2
Triglycerides, free fatty acids, and diglycerides	57
Other components	3

The efficiency of gastrointestinal absorption of squalene has been estimated at 85% in humans [10] and it is distributed from the gastrointestinal tract to various tissues [6]. Squalene concentrations in more than 25 human tissues also varied widely; the highest levels were in skin (about 475 μg/g dry weight) and adipose tissue (about 275 μg/g), whereas only moderate amounts were found at sites of active cholesterol synthesis (liver, 75 μg/g; small intestine, 42 μg/g).

Only a very small amount of squalene taken up as a nutrient is converted to cholesterol, and higher consumption of squalene does not change the cholesterol level [11]. The average daily dietary intake of squalene in Mediterranean countries is in the range of 200–400 mg/day [12]. Squalene is considered a remarkable bioactive substance with several interesting biological activities. Increased amounts of squalene in the serum are safe, beneficial, and exhibit antioxidant, chemopreventive, antitumor, and hypocholesterolemic properties [13–16]. Interestingly, squalene is one of the predominant components (about 13%) of human sebum (Table 1). It seems to be critical for reducing free radical oxidative damage to the skin and thus for maintaining skin health.

BIOLOGICAL ACTIVITIES OF SQUALENE

As mentioned above, squalene is a polyunsaturated triterpene comprising six isoprene units possessing antioxidant properties. Squalane is a saturated derivative of squalene (Figure 2). Squalene is structurally similar to carotenoids (β-carotene and lycopene) and is an intermediate metabolite in the synthesis of cholesterol and other steroids (Figure 3). In plants, squalene cyclizes to form phytosterols, whereas in animals cyclization to cholesterol takes place [17].

To date, anticancer, antioxidant, drug carrier, hypocholesterolemic, detoxifying, skin hydrating, and emollient activities of squalene have been reported. Thanks to these properties, squalene is especially useful for use in nutrition, pharmaceuticals, cosmetics, and medicine [18].

Skin and Eyes

As mentioned above, squalene is one of the main components of SSLs and a key component in maintaining skin health. Certainly, squalene seems to function in the skin as a quencher of single oxygen, protecting the human skin surface from

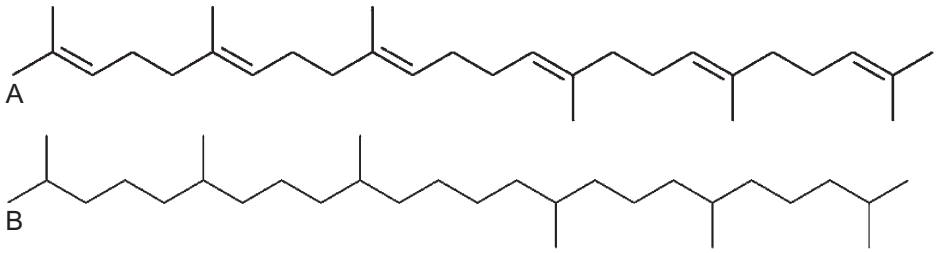

FIGURE 2 Chemical structure of squalene (a) and squalane (b).

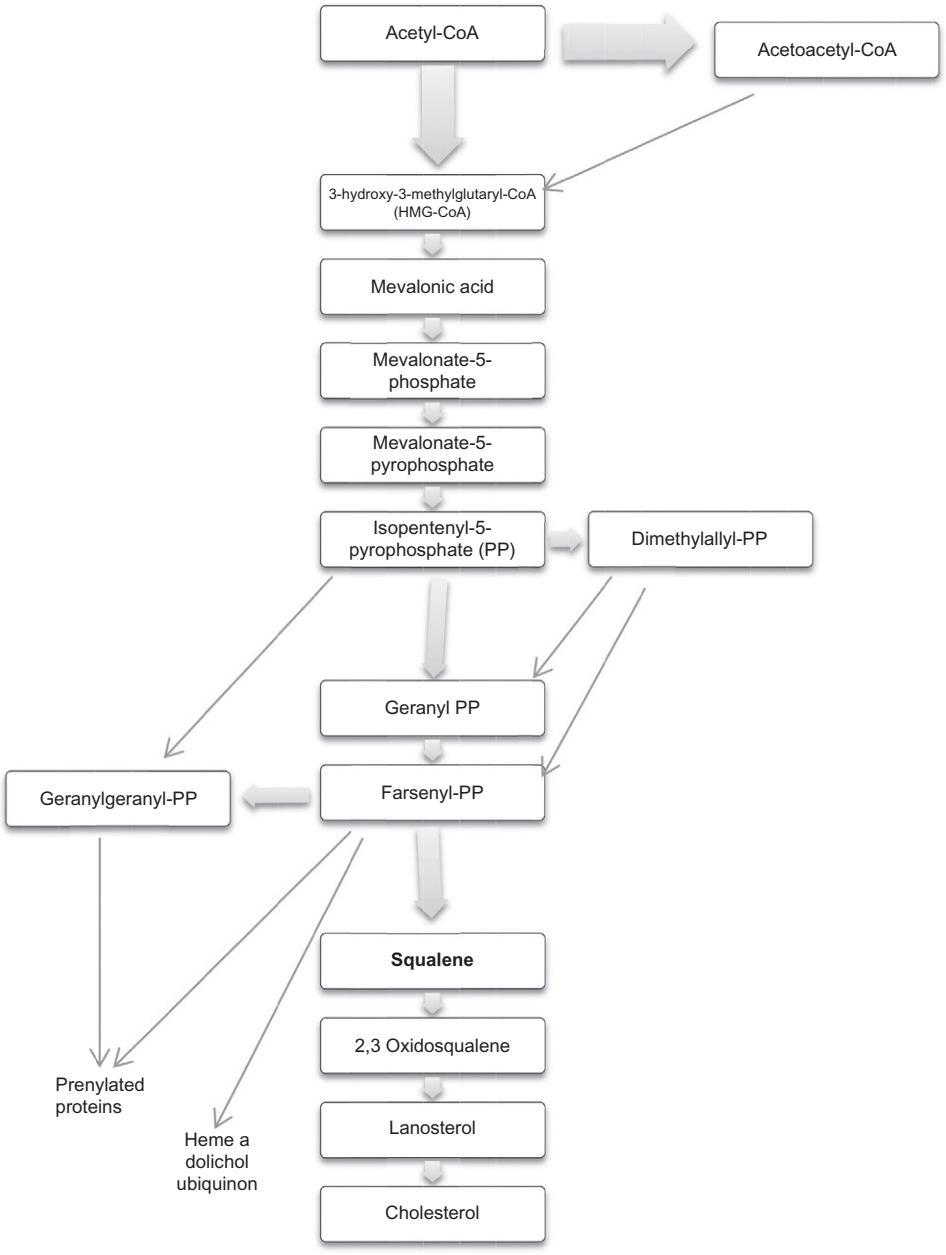

FIGURE 3 Cholesterol metabolic pathway. The schematic representation of the cholesterol biosynthetic pathway includes the squalene, a precursor of cholesterol.

lipid peroxidation caused by exposure to ultraviolet (UV) light and other sources of oxidative damage [19]. SSLs form the first line of defense against the potential danger induced by the UV components of solar light (UVB and UVA). One of the major photoprotective components of SSLs is squalene; working as antioxidant, it blocks photo-induced lipid peroxidation in cellular skin components by quenching singlet oxygen [20]. Kohno et al. [19] reported that squalene is both a highly effective oxygen-scavenging agent and resistant to peroxidation, remaining stable against attacks by peroxide radicals. Thus, adequate levels of squalene on the surface of human skin could arrest the propagation of the chain reaction of lipid peroxidation.

On the basis of all described biochemical properties, we can hypothesize that squalene is capable of neutralizing reactive oxygen species (ROS) induced by UV irradiation on the skin; it may also prevent the corresponding lipid peroxidation at the surface of human skin. The presence of squalene in human sebum can be considered an evolutionary advantage. This affirmation is supported by the fact that squalene is unique to human sebum and is completely missing in the main genera of nonhuman primates, including those closer to man, the *hominoidea*. The skin of monkeys, unlike that of humans, is effectively covered by a large quantity of hair, protecting the animal from UV rays. In opposition, squalene concentration in adult human skin reaches up to 20% [21].Consequently, in the far less hairy human skin, the protective function could be reasonably carried out by squalene. Alternatively, squalene is also a principal surface lipid of different semiaquatic mammals, namely otter and beaver, among others [22]. In these species, squalene accounts for the essential properties of water repellence and thermal insulation.

In view of the physiological relevance of squalene in skin photoprotection, the possibility of controlling SSL composition by dietary intervention is a promising perspective. Even more interesting, some authors have shown that skin lipid film can be modulated through the diet more efficiently that through topical application [23]. In humans, dietary squalene is absorbed, transported in serum, and distributed ubiquitously in human tissues; the greatest accumulation occurs in the skin through sebocyte concentration [13]. It has been convincingly demonstrated that dietary bioactive compounds have beneficial effects on skin health [24,25]. Cho et al. [26] reported that in healthy volunteers, high oral dosage (>13.5 g/day) of squalene significantly decreased wrinkles in aged human skin, increased type I procollagen, and decreased UV-induced deoxyribonucleic acid (DNA) damage. Therefore we can speculate that the regular consumption of virgin olive oil rich in squalene may help maintain healthy skin. On the other hand, squalene is described as a great emollient, and it is quickly and efficiently absorbed deep into the skin, restoring its suppleness and flexibility. Interestingly, it has been reported that oral supplementation of squalene in mice resulted in a marked dose-dependent upregulation of cellular and nonspecific immune functions [13]. Remarkably, squalene also seems to play an important role in the health of the retina, with particular regard to reducing free radical oxidative damage in rod photoreceptor cells [27]. In general, we could conclude that a diet with an adequate intake of oils containing squalene, such as virgin olive oil, might be sufficient to achieve the protective benefits described above.

Serum Cholesterol Concentration

Squalene is an important intermediate in the endogenous synthesis of cholesterol. Thus one could argue that administration of squalene could increase serum cholesterol concentrations and enhance the risk for the development of atherosclerosis. However, it has been reported that squalene has no effect or even decreases serum cholesterol concentrations. Rao et al. [28] described that dietary administration of 1% squalene over a 10-week period did not increase serum cholesterol concentrations. Strandberg et al. [11] reported that daily dietary intake of 900 mg of squalene for a period of 7–10 days in humans produced a 17-fold increase in serum squalene, but serum triglyceride and cholesterol contents were unchanged. Even more interesting, Chan et al. [29] described that a combination therapy (pravastatin and squalene) administered to patients with hypercholesterolemia significantly reduced total cholesterol and LDL cholesterol and increased high-density lipoprotein (HDL) cholesterol. These effects induced by squalene may be due to increased fecal elimination of cholesterol as fecal bile acids and inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by dietary squalene-derived cholesterol synthesis.

Cardiovascular Disease

Observational cohort studies and a secondary prevention trial showed an inverse association between adherence to the Mediterranean diet and cardiovascular risk [2,30,31]. In addition, olive oil has been shown to improve cardiovascular risk factors. A recent primary prevention trial conducted by Estruch et al. [4] determined that among people at high cardiovascular risk, a Mediterranean diet supplemented with extra virgin olive oil significantly reduced the incidence of major cardiovascular events: myocardial infarction, stroke, or death from cardiovascular causes. They concluded that extra virgin

olive oil is probably responsible for most of the observed benefits of Mediterranean diets. These results support the benefits of both the Mediterranean diet and virgin olive oil in the primary prevention of cardiovascular disease. Recent studies have reinforced the proposed mechanisms by which virgin olive oil can exert its beneficial effects on cardiovascular risk, including (1) improvement of the lipid profile through a decrease in total and LDL cholesterol and an increase of the HDL-to-cholesterol ratio; (2) reduction of the susceptibility of LDL to oxidation and amelioration of oxidative vascular damage; (3) improved endothelial function; (4) improved blood pressure control; and (5) favorable modifications of hemostasis [32]. Some of these beneficial effects can be attributed to so-called minor compounds. As mentioned earlier, squalene represents the main minor component of virgin olive oil. Thus, it is interesting to know whether squalene has a role in the development of cardiovascular diseases. Previous studies demonstrated that small amounts of this compound were present in normal aorta and in atherosclerotic plaque of humans and rabbits [33]. These findings generate an open discussion regarding its role at these sites because atherosclerosis is the main cause of all manifestations of cardiovascular diseases. Kritchevsky et al. [34] described the experimental administration of a diet containing 3% squalene for 7 weeks to rabbits, showing the absence of changes in atheroma development. Guillen et al. [35] showed that squalene feeding reduced atherosclerotic lesion size in apolipoprotein E-deficient male mice. Likewise, Bullon et al. [36] published findings showing that squalene administration also reversed endothelial activation and lowered cellularity in gingival mucosa of atherosclerotic rabbits. Another interesting article described that squalene ameliorates atherosclerotic lesions through the inhibition of oxidized LDL uptake by macrophages by reducing CD36 scavenger receptor expression in macrophages [37]. These data suggest that squalene administration could be a safe and useful alternative in the management of atherosclerotic disease. Nevertheless, these results need to be confirmed in human trials before being considered an important contributor to the cardiovascular protective effect attributed to virgin olive oil. Moreover, a cardioprotective action of squalene also has been reported. Indeed, administration of a diet containing 2% squalene for 45 days effectively prevented isoproterenol-induced myocardial infarctions in male albino rats [38]. Motawi et al. [39] also reported that squalene acts as a cytoprotectant capable of attenuating cyclophosphamide-induced alterations in rat myocardium. Taken together, these findings suggest that squalene is a promising agent in both the prevention and management of cardiovascular disease [33].

Cancer

Positive associations between increased intake of dietary fat and cancer of the colon, breast, prostate, and ovary have been shown. However, evidence suggests that it is not only the amount but also the type of dietary fat that is important in the etiology of some cancers. In fact, epidemiological data show that high consumption of virgin olive oils, which are particularly rich in squalene, should afford considerable protection against cancer [15,40]. Thus, in the Mediterranean countries where virgin olive oil is the main cooking and garnishing fat used, the intake of squalene in the diet is high when compared with other regions. In fact, the average daily dietary intake of squalene in Mediterranean countries is in the range of 200–400 mg/day [12], whereas in the United States intake has been estimated to be only about 30 mg/day [6]. Consequently, squalene is consumed as an integral part of the human diet. Newmark [41] suggested that the lower risk of some cancers associated with virgin olive oil consumption might be attributable to squalene. Interestingly, it has been claimed that sharks, which have high levels of squalene in tissue, are resistant to cancer. Thus squalene is believed to be partially responsible for the low incidence of human cancer in the Mediterranean region, where consumption of virgin olive oil is high [15].

Experiments in rodents suggest that squalene exhibits antitumor activity against skin, colon, and lung cancer as well as sarcoma [28,42–44]. The mechanism proposed to explain the activity of squalene is based on its strong inhibitory action on β-hydroxy-β-methylglutaryl-coenzyme A reductase catalytic activity *in vivo*. This activity affects Ras p21 farnesylation, signal transduction, and cellular proliferation [41].

Squalene also has been reported to possess antioxidant properties. Oxidative stress may result in an increase in oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant system. ROS can damage cellular macromolecules, including DNA, and this damage is directly responsible for carcinogenesis. Oxidative stress is considered one of the major factors causing different diseases such as cancer and atherosclerosis. Considering that oxidative stress is involved in the pathophysiology of all cancers [45], the prevention of oxidative stress in human cells could be a suitable way to prevent the development of cancer.

Antioxidant nutrients play a significant role in the body's defense against excess levels of free radicals and prevent the onset of degenerative diseases. In particular, antioxidants that can be stored in cellular membranes may be potential candidates for the prevention of disorders involving oxidative damage during disease progression. Tilvis et al. [46] reported that adipocyte squalene could be segregated anatomically and functionally into two distinct pools: a metabolically active pool in the microsomal membranes and a metabolically inactive pool in fat droplets. In this context, squalene has been reported to possess both antioxidant and membrane-stabilizing properties. Squalene has been considered to be an

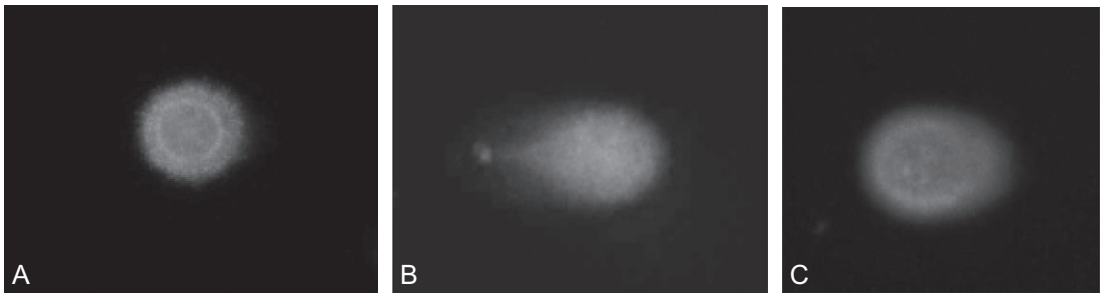

FIGURE 4 Representative images of Comet assay analysis of MCF10A (human mammary epithelial cells) untreated cell (a); 10 min H_2O_2 exposed cell (b) and; 10 min H_2O_2 exposed after 24 h Squalene 200 μM pretreated cell (c). (a) Cell shows a circular form indicating absence of DNA damage. (b) Cell exhibits a long and high bright tail related to the DNA strand breaks and indicating an important DNA oxidative damage. (c) Cell illustrates the reduction of tail length and fluorescent intensity indicative of the reduction of DNA damage.

antioxidant exerting anticarcinogenic activity by enhancing cellular antioxidant status [14,42]. It has been described that squalene reduces *in vitro* ROS levels and protects against oxidative DNA damage in human mammary epithelial cells (Figure 4) but not in breast tumor cells [16].

Overall, the results suggest that squalene acts as an antioxidant only on mammary epithelial cells. Therefore we can speculate that squalene might contribute to the preventive effect of olive oil against human breast cancer by inhibiting oxidative stress. Moreover, these *in vitro* results suggest that squalene may play a role in the prevention of human breast cancer, but it is probably ineffective once breast tumors are established [16]. Significantly, virgin olive oil consumption decreased the incidence of breast cancer [47]. This selective effect of squalene also was described in other cell types. Das et al. [48] found that squalene decreased ROS levels in bone marrow cells but not in neuroblastoma cells. The mechanism of such selective antioxidant sensitivity is unknown, but we may consider the following possibilities: (1) it is possible that squalene selectively increases glutathione levels in normal cells but not in cancer cells, a phenomenon described as the "GSH paradox" [49]; (2) the selective sensitivity might be related to differences in cellular uptake and accumulation of squalene or the status of the mevalonate pathway [48]; (3) the selective sensitivity might be related to the differential regulation of antioxidant systems in normal versus tumor cells [50].

One of the major photoprotective components of SSLs is squalene working as an antioxidant; it blocks photo-induced lipid peroxidation in cellular skin components by quenching singlet oxygen [19]. Experimental studies have shown that squalene can effectively inhibit chemically induced skin tumorigenesis in rodents [14]. Squalene has a major protective effect against skin cancer, probably by scavenging singlet oxygen generated by UV light [51]. The oral intake as well as the external use of olive oil have been shown to provide photoprotection to the skin [13].

Thus we may hypothesize that because of the high concentrations of squalene in virgin olive oil, which is transferred to the skin, virgin olive oil intake could be useful to protect against skin cancer. Nevertheless, although animal studies showed the action of squalene in decreasing carcinogenesis, one should be cautious in extrapolating those findings to humans. Further experiments to fully evaluate this natural compound for its cancer-preventive properties in humans are warranted. Table 2 summarizes the biological activities of squalene described in this chapter.

SUMMARY POINTS

- The Mediterranean diet is a well-known model of diet for the prevention of major chronic diseases. These findings are relevant in terms of public health and are in agreement with current guidelines and recommendations from all the major scientific associations that strongly encourage a Mediterranean-like dietary pattern for the primary and secondary prevention of major chronic diseases.
- Virgin olive oil is considered as a key component of the Mediterranean diet and is the main source of dietary lipids. Based on the scientific evidence, virgin olive oil can be categorized as a functional food not only because of its balanced lipid composition but also mainly because of the presence of so-called minor components, among which is squalene.
- Squalene represents the main minor component of virgin olive oil; therefore it is an important component in the diet of Mediterranean peoples. Squalene is a naturally occurring lipid component present in healthy diets, and it is recognized as a functional compound of high importance because of its beneficial effects on human health.
- Squalene could be partially responsible for the health benefits attributed to virgin olive oil intake.
- Squalene has several beneficial properties: it is a natural antioxidant, it decreases the serum cholesterol concentration, and it possesses photoprotective, tumor-protective, and cardioprotective properties.

TABLE 2 Summary of the Biological Activities of Squalene Described

Location	Activity	Species	References
Skin	Protection against lipid peroxidation	Human	[19]
	Antioxidant	Human	[20]
	Reduction of wrinkles	Human	[26]
	Antitumor activity	Mouse	[42]
	Inhibition of tumorigenesis	Rodent	[14]
	Protection against cancer	Human	[51]
Blood	Decrease of serum cholesterol levels	Rat, human	[11,28]
	Reduction of total cholesterol and LDL cholesterol	Human	[29]
	Increase of HDL cholesterol	Human	[29]
	Reduction/amelioration of atherosclerotic lesion size	Mouse, human	[35,37]
Sarcoma	Antitumor activity	Mouse	[43]
Lung	Antitumor activity	Mouse	[44]
Colon	Antitumor activity	Rodent	[28]
Gingival mucosa	Reversion of endothelial activation	Rabbit	[36]
Retina	Reduction of free radical oxidative damage	Bovine/frog	[27]
Myocardium	Cytoprotection	Rat	[39]
Adipocyte	Antioxidant and membrane-stabilizing properties	Human	[46]
Breast	Protection against oxidative DNA damages	Human	[16]
	Reduction of ROS levels	Human	[16]
Bone marrow	Reduction of ROS levels	Mouse	[48]

- In the Mediterranean countries where virgin olive oil is the cooking and garnishing fat of choice, intake of squalene in the diet is likely to be considerably higher than in other areas of the world. Thus adequate intake of virgin olive oil provides a continuous supply of squalene that is currently linked to the prevention of diseases such as cancer and cardiovascular disease.

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Bioactive Properties of the Main Triterpenes Found in Olives, Virgin Olive Oil, and Leaves of Olea Europaea

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- Trabajo enviado a la revista *"Journal of Agricultural and Food Chemistry".*
- Fecha de publicación: Noviembre de 2013
- Nº de citas (a 1 de Diciembre de 2015): 15
- SCImago Journal & Country Rank
  - Categoría perteneciente: Agricultural and Biological Sciences
  - Índice SJR: 1,148
  - H index: 199
  - Posición: 23/204
  - Cuartil en la categoría: Q1
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Food Sciences and Technology
  - Índice de impacto: 2,912
  - Posición: 13/123
  - Cuartil en la categoría: Q1

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

pubs.acs.org/JAFC

Bioactive Properties of the Main Triterpenes Found in Olives, Virgin Olive Oil, and Leaves of *Olea europaea*

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ABSTRACT: Oleanolic acid, maslinic acid, uvaol, and erythrodiol are the main triterpenes present in olives, olive tree leaves, and virgin olive oil. Their concentration in virgin olive oil depends on the quality of the olive oil and the variety of the olive tree. These triterpenes are described to present different properties, such as antitumoral activity, cardioprotective activity, anti-inflammatory activity, and antioxidant protection. Olive oil triterpenes are a natural source of antioxidants that could be useful compounds for the prevention of multiple diseases related to cell oxidative damage. However, special attention has to be paid to the concentrations used, because higher concentration may lead to cytotoxic or biphasic effects. This work explores all of the bioactive properties so far described for the main triterpenes present in virgin olive oil.

KEYWORDS: *Olea europaea, virgin olive oil, antioxidant activity, oleanolic acid, maslinic acid, uvaol, erythrodiol, cancer, cardiovascular, inflammation, oxidative stress*

INTRODUCTION

Nowadays there is an increasing interest in healthy eating habits and physical care to improve our health and quality of life. In fact, government and educational agencies are trying to re-educate the eating habits of the population.

Mediterranean habits are known to be among the healthiest to improve age-dependent vascular activity,[1] and they have proved to be beneficial for several diseases such as the metabolic syndrome or coronary heart disease.[2,3]

Mediterranean habits include exercising regularly and following the so-called Mediterranean diet. This diet consists of bread, cereal, rice, pasta, fruits, and vegetables mainly and olive oil as the principal source of fat.[4] Virgin olive oil has been described to possess bioactive properties such as cardioprotective effects, commonly associated with high levels of monounsaturated fatty acids (MUFA),[5] but these effects would not necessarily be promoted by MUFA alone. Antioxidant and antiatherogenic activities,[6] antiproliferative and pro-apoptotic capacities on human cancer cell lines,[7,8] protection against oxidative DNA damage,[9] and anti-inflammatory properties[10] have been described mostly in its minor compounds. Virgin olive oil is composed by triacylglycerides and 1−2% of minor components (about 230 different compounds). It can be divided into two fractions, the unsaponifiable fraction, extracted with solvents after the saponification of the oil, and the saponifiable fraction. In the unsaponifiable fraction of virgin olive oil there are triterpenic alcohols and other pentacyclic triterpenes, which together form the main triterpenes of virgin olive oil.

However, very little is known about the activity of this group of compounds, known as triterpenes, present in the leaves and skin of olives and in virgin olive oil, too.[11] In this paper, we summarize the effects of the major triterpenes present in virgin olive oil described so far, and the key factors of their action regarding their role in the oxidation mechanism of the cell.

OLIVE OIL TRITERPENES

The main triterpenes of virgin olive oil are oleanolic acid, maslinic acid, uvaol, and erythrodiol.[11] These two hydroxyl pentacyclic triterpene acids (oleanolic and maslinic acid) and these two dialcohols (uvaol and erythrodiol) are differentiated according to the function present at the C-17 position. Maslinic acid has two vicinal hydroxyl groups at the C-2 and C-3 positions, besides the carboxyl radical. Uvaol and erythrodiol possess two hydroxyl groups in remote positions and are different with regard to the methyl group location (Figure 1). These triterpenes are found in olive skin and the leaves of olive trees (*Olea europaea*). The Picual variety showed the highest content of triterpenes in olives. The various types of commercial black and green olives ranged from 460 to 1470 mg/kg fruit. Natural black olives, not treated with NaOH (which debitters black and green olives for commercial treatments), showed concentration >2000 mg/kg in the olive flesh.[107] The leaf contains important amounts of oleanolic acid (3.0−3.5% DW), followed by maslinic acid and minor levels of erythrodiol and uvaol. The content of triterpenoids changes during leaf ontogeny.[108] Otherwise, in virgin olive oil, the concentration oscillated between 8.90 and 112.36 mg/kg.[11] Allouche et al. concluded that the high

Received: July 19, 2013
Revised: October 31, 2013
Accepted: November 18, 2013
Published: November 22, 2013

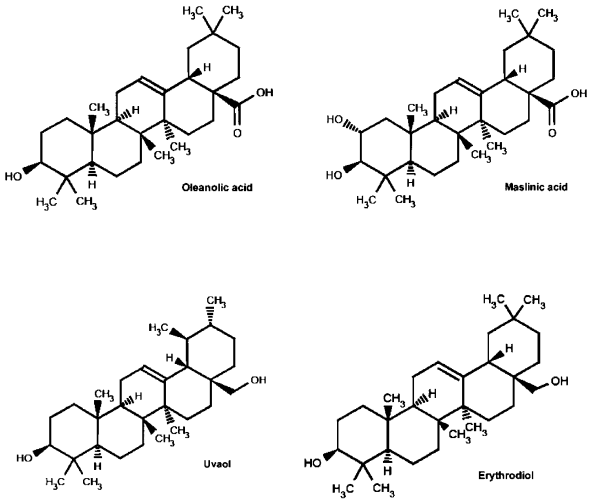

Figure 1. Chemical structures of oleanolic and maslinic acids and uvaol and erythrodiol dialcohols of olive oil.

variability observed in virgin olive oil triterpenic composition was due to genetic factors. High triterpenic content was obtained from 4 of the 40 varieties (Lechin de Granada, Dolce Agogia, Cornicabra, and Salonenque).[11] Other authors point out that the concentration of hydroxyl pentacyclic triterpene acids depends on the quality of olive oil.[12] Finally, it is recognized that triterpene concentration varies depending on the type of cultivation and the handling of olive oil. In fact, triterpenes are present in higher concentrations in olive pomace oil than in virgin olive oils.[12]

Very few papers describe the bioavailability of pentacyclic triterpenes from virgin olive oil intake, but some reveal interesting data from bioavailability in humans and rats.

Oleanolic Acid. After oral administration of 50 mg/kg to rats, a maximum concentration of 0.29 ± 0.26 μM was observed at 21 ± 17 min; oleanolic acid was minimally absorbed, with an absolute oral bioavailibity of 0.7%.[103] In humans, the plasmatic concentrations determined in healthy male volunteers after a single oral administration of 40 mg was 26.5 ± 15 nM at 5.2 ± 2.9 h.[104] Rada et al.[106] show that oleanolic acid and human serum proteins have molecular interactions between them, and these serum proteins are known for the important role in the binding of basic and neutral drugs. These authors demonstrated the formation of complexes between human serum proteins and OA.

Maslinic Acid. Maslinic acid after a single oral administration (50 mg/kg) to rats is absorbed in the intestine and reaches the blood, where it is found 10 min after the oral administration and can still be detected in plasma after 60 min.[105]

Uvaol and Erythrodiol. For uvaol and erythrodiol, we have not found in the literature consulted any reference concerning bioavailability.

Another important issue is the concentration used by the different authors in all of the different studies. Sánchez-González et al.[109] described an interesting property of maslinic acid, namely, its safety. In this paper, authors examined the administration of 50 mg/kg of maslinic acid for 28 days and a single oral administration of 1000 mg/kg to mice. Their results show that this compound does not produce any adverse effects on the variables tested in mice (morbidity, mortality, toxicity, body weight...), suggesting its use as a nutraceutical. We have to pay attention to the concentrations used with each triterpene because Lu et al. described another aspect of oleanolic acid, its hepatotoxic effect in mice in a dose-dependent manner. Oleanolic acid causes body weight loss, inflammation and hepatocellular apoptosis, necrosis, and feathery degeneration (indicative of cholestasis)[14] in mice. Depending on the concentration used and exposure time, the effect could be contrarily adverse or even have a biphasic effect; for example, Allouche et al.[6] observed an antithrombotic effect after 10 min of incubation period and a prothrombotic effect when the incubation period was prolonged to 20 min, which was attributed to alteration of maslinic acid after an extended exposure time with prothrombinase complex. Marquez-Martin et al. showed that the behavior of oleanolic acid and uvaol was suggestive of a biphasic response in terms of TNF-α production. There was an increase at low concentrations (10 μM) and a decrease at higher ones (100 μM).[15] Therefore, it seems that depending on the concentration and time used, these triterpenes have different effects on body response. Consequently, more studies are needed about the bioavailability and metabolism of these triterpenes with virgin olive oil and olive ingestion to be able to assess the potential effect that they could have with diet intake. Future studies should be adapted to the bioavailability concentration found for each triterpene; in this way the results obtained could be more reliable.

BIOACTIVE PROPERTIES OF OLIVE OIL TRITERPENES

In the past years, there have been a growing number of studies focusing on the activity of the virgin olive oil triterpenes. Antitumoral, anti-inflammatory, antioxidant, hepatoprotective, cardioprotective, and antimicrobial activities have been recently described.[6,7,16−19] Here we analyze the different bioactivities of these compounds against different diseases and conditions and future possible applications.

Cardiovascular Disease. Some of the risk factors of cardiovascular diseases are age, sex, and genetic makeup. These are not modifiable, but there are other risk factors that could be altered. Among the modifiable risk factors the following, among others, should be included: levels of high-density lipoprotein (HDL) cholesterol, levels of low-density lipoprotein (LDL) cholesterol, obesity, tobacco, levels of circulating oxidized LDL, hypertension, endothelial dysfunction, and oxidative stress, among others. Nowadays, a high number of myocardial heart attacks could be prevented by these modifiable factors, which are influenced by the diet.[20] The diet followed is responsible, to a greater or lesser extent, for atherosclerosis. Atherosclerosis is an oxidative, inflammatory, and thrombotic disease characterized by the deposition of lipid and other bloodborne material within the arterial wall of almost all vascular territories, which is the prelude to atheroma emergence.[29]

The connection between high levels of LDL oxidation and the increase in cardiovascular disease risk[21−23] and an early event in atherosclerosis has already been described.[24] Several studies interconnect certain foodstuffs and the oxidation process of LDL,[25] so that it seems that diet and cardiovascular disease are strongly linked. Indeed, at present, diet is considered an important determinant in the prevention of cardiovascular diseases.[26] In this way, triterpenes may play a key role in decreasing this LDL oxidation and, hence, in decreasing cardiovascular disease incidence. Oxidation of LDL may play a critical role in the early stages of the disease, whereas thrombosis acts at the latest stages, it being one of the fatal clinic consequences of this pathology.[30] Apart from preventing LDL oxidation, these compounds have been described as antiathero-

Table 1. Bioactive Properties of the Main Triterpenes Found in Olives, Olive Tree Leaves, and Virgin Olive Oil in Cardiovascular Disease

Cardiovascular Disease	Triterpene	Action	Doses	Assay	Reference
	Oleanolic acid	Protection against LDL oxidation	10 - 20 μM	*In vitro*	27, 28
		Antiatherogenic	100 mg/kg/day	*In vivo (*apoE knouckout mice) 8 weeks of treatment	31
		Antihyperlipidemic and antihypertensive	60 mg/kg/day	*In vivo* (DSS rats) 6 weeks of treatment	37
		Hypoglycemic effect	60 mg/kg/day	*In vivo* (DSS rats) 6 weeks of treatment	37
		Antioxidant and nitric oxide releasing action	60 mg/kg/day	*In vivo* (Wistar rats) 5 weeks of treatment	39
		Vasorelaxation in aortic rings	orujo oil intake	*In vivo* (rats)1 dosage	40
		Endothelium-dependent release of NO	3 - 30 μM	*In vivo* (Wistar rats) 12-16 weeks old rats	41
	Maslinic acid	Inhibition of LDL oxidation	12.5 – 400 μM 10 - 20 μM	*In vitro*	6,28
		Cardioprotective	15 mg/kg	*In vivo* (Wistar rats) 7 days of treatment	33
	Uvaol	Protection against LDL oxidation	10 - 20 μM	*In vitro*	28
		Antiatherogenic	12.5 – 400 μM	*In vitro*	6
		Cardiac hypertrophy reduction and left ventricle remodelling	50 mg/kg/day	*In vivo (*mice) 2 weeks of treatment	42
	Erythrodiol	Antiatherogenic	12.5 – 400 μM	*In vitro*	6
		Vaxorelaxation in aortic rings	orujo oil intake	*In vivo* (rats) 1 dosage	40
		Cardiac hypertrophy reduction and left ventricle remodelling	50 mg/kg/day	*In vivo* (mice) 2 weeks of treatment	42

genic, because of the role that the triterpenic diols, uvaol and erythrodiol, play in preventing LDL-supporting thrombin generation in vitro.[6] Table 1 shows triterpene actions in the different stages of the development of cardiovascular disease.

Oleanolic Acid. Oleanolic acid was described as playing a protection role against LDL oxidation like other triterpenes with the effect of chiosmastic gum (CMG), the most effective protecting human LDL oxidation yet known.[27,28] Thus, oleanolic acid exerts potent antiatherogenic effects independent of plasma lipid levels in apolipoprotein E knockout mice.[31] Previous studies of isolated oleanolic acid describe its action in preventing hypertension and hyperlipidaemia in Dahl salt-sensitive (DSS) rats with genetic hypertension. In this study, oleanolic acid is described as preventing the development of severe hypertension through its potent diuretic−natriuretic−saluretic activity, its direct cardiac effect, and its antihyperlipidemic, antioxidant, and hypoglycemic effects on DSS rats.[37] Other authors show its possible action in inhibiting the progress of fibrosis and in decreasing the portal pressure in CCl_4-induced portal hypertensive rats, which could be related to the increase of eNOS expression and enhancement of nitric oxide (NO) level in the liver.[38] This prevention of hypertension has also been attributed to the antioxidant and nitric oxide releasing action of oleanolic acid.[39] Indeed, Rodríguez-Rodríguez et al. showed how oleanolic acid together with erythrodiol was able to promote vasorelaxation in aortic rings with endothelium precontracted in rats.[40] This effect seemed to be mainly mediated by endothelial production of NO. Later, this effect was studied,[41] and oleanolic acid was shown to activate endothelium-dependent release of NO and to decrease smooth muscle cell calcium followed by relaxation. This oleanolic acid-evoked endothelium-derived NO release was independent of endothelial cell calcium and involved phosphoinositide-3-kinase-dependent phosphorylation of Akt-Ser(473) followed by phosphorylation of eNOS-Ser(1177).

Oleanolic acid is also involved in atherosclerosis protection also, with antihyperlipidemic effects in Wistar rats, decreasing hepatic expression levels of lipogenic genes, and several cytochrome P450 genes.[32]

Maslinic Acid. Maslinic acid strongly inhibits in vitro LDL oxidation.[6] However, maslinic acid showed both pro- and antithrombotic effects depending on the concentration used.[6] Thus, special attention has to be paid to the concentration of these compounds employed, because depending on that, the effects could change, which has been already described above. Another cardioprotective activity described for maslinic acid was its effect on isoproterenol-induced myocardial infarcted albino Wistar rats; maslinic acid reduced the effects of isoproterenol on body weight, heart weight, lipids, lipoproteins, lipid peroxidation, cardiac marker enzymes, and paraoxonase,[33] so it that seems maslinic acid has cardioprotective effects, influencing more than one pathway.

Consequently, maslinic acid may act both at the beginning and at the latest stage of atherosclerosis. Indeed, it has been described that this compound has been shown to be involved in atherosclerosis protection, with potential antioxidant and hypoglycemic effects by reducing insulin resistance in a mouse model of genetic type-2 diabetes.[32] However, more studies are needed to evaluate the precise mechanism of action of these compounds in atherosclerosis prevention.

Table 2. Bioactive Properties of the Main Triterpenes Found in Olives, Olive Tree Leaves, and Virgin Olive Oil in Cancer

Cancer	Triterpene	Action	Doses	Assay	Reference
	Oleanolic acid	Invasion and migration decrease, ROS decrease, NO decrease, VEGF expression decrease	2 - 4 μM	*In vitro* (human liver cancer cells)	43
		Antitumoral activity	10 - 100 μM	*In vitro* (skin, hepatocellular, colon, lung, breast, pancreatic cancer cell lines and myelogenous leukemia)	7, 43-48
		Apoptosis induction by mitochondrial pathway	12,5 – 200 μM	*In vitro* (hepatocellular carcinoma and human pancreatic cancer cell line)	44, 45, 53, 54
		Cell cycle arrest	0 - 50 μg/ml	*In vitro* (hepatocellular carcinoma and human pancreatic cancer cell line)	44, 54
		Inhibit proliferation and colony formation. Apoptosis by mTOR signaling	12.5 – 100 μM	*In vitro* (osteosarcoma cells)	59
		Apoptosis by p53, Bax, Bcl-2 and caspase-3	2, 4 or 8 μM	*In vitro* (melanoma, colon and liver cancer cells)	55-58
	Maslinic acid	Invasion and migration decrease, ROS decrease, NO decrease, VEGF expression decrease	2 - 4 μM	*In vitro* (human liver cancer cells)	43
		Antitumoral	0 -100 μM	*In vitro* (skin, hepatocellular, colon, lung, breast, pancreatic cancer cell lines and myelogenous leukemia)	7, 43-48
		Chemopreventive	3.75 – 30 μM 100 mg/kg/day	*In vitro* (colorectal cancer)& *In vivo* (6 weeks of treatment)	46, 60
		Suppression of COX-2 expression, NFκβ and AP-1 inhibition	Unknown	*In vitro* (Raji cells)	61
		Antimetastatic activity	0-25 μM	*In vitro* (DU145 human prostate cancer cell line)	62
		Apoptosis induction through caspase 3	0 -100 μM	*In vitro* (different cancer cell lines)	63-67
		Suppression of NFκβ	0 – 50 μM	*In vitro* (pancreatic cancer cell line)	68
	Uvaol	Pro-apoptotic potential through JNK activation	0 -100 μM	*In vitro* (breast cancer cell, astrocytoma cells)	7,74,76
		Pro-apoptotic associated to ROS	0 -100 μM	*In vitro* (human breast cancer cells,	7, 76
		Antitumoral	0 -100 μM	*In vitro* (murine and human cancer cell lines)	7, 73-76
		Inhibition of proliferation	0 – 300 μM	*In vitro* (gastric cancer cell line)	70
	Erythrodiol	Pro- apoptotic potential	0 - 100 μM	*In vitro* (breast cancer cells, colon cancer cells astrocytoma cells)	7, 72,74, 76
		Antitumoral	0 -100 μM	*In vitro* (murine and human cancer cell lines)	7, 73-76
		Antiproliferative	0 – 150 μM	*in vitro (colon cancer cells)*	72

Uvaol and Erythrodiol. The action of erythrodiol and uvaol in reducing cardiac hypertrophy and left ventricle remodeling induced by angiotensin II in mice, through diminishing fibrosis and myocite area, has been recently described. They seem to

modulate growth and survival of cardiac myofibroblasts, and both of them inhibit the angiotensin II-induced proliferation in a PPAR-γ-dependent manner, whereas at high doses they activate pathways of programmed cell death that are dependent on JNK and PPAR-γ.[42]

There are several studies on olive pomace oil, which has high triterpenic content, and on its improvement of the endothelial function,[34−36] so it seems reasonable to conclude that these compounds could have an active role in cardiovascular prevention.

In view of the actions described above, virgin olive oil triterpenes could have an interesting therapeutic potential as cardiovascular drugs, and furthermore they may fulfill a role in preventing, through diet, different kinds of cardiovascular disorders. Although more evidence will be necessary to identify the mechanism involved and their interactions, it will be necessary to determine the most effective dose and exposure time for treatments.

Cancer. A diversity of studies highlight different aspects of the function that triterpenes seem to play in cancer. So far, it is clear that triterpenes affect tumorigenesis and key factors for its development, such as angiogenesis.[43] Apart from this, various studies note the antitumor activities of triterpenes in different cancers such as hepatocellular carcinoma, skin cancer, colon cancer, lung cancer, breast cancer, myelogenous leukemia, and pancreatic cancer.[7,13,44−49]

In this line, the antiangiogenic effects of oleanolic and maslinic acids in human liver cancer cell lines have been studied. In a dose-dependent manner they reduced cell invasion and migration, decreasing reactive oxygen species (ROS) and NO levels and decreasing expression of vascular endothelial growth factor (VEGF).[43] In Table 2 the kinds of action exerted by each triterpene in the different types of cancer studied are specified.

Several studies have focused on the antitumoral activity of these triterpenes in the synthesis of new molecules derived from them and assessed their roles as anticancer drugs.[50−52]

Oleanolic Acid. The mechanism of action of oleanolic acid has been studied in different types of cancer cells. On hepatocellular carcinoma, oleanolic acid exhibited inhibitory effects through induction of apoptosis and cell cycle arrest.[44,53] Apoptosis was induced through the mitochondrial pathway, and this could be due to ROS generated by mitochondrial fatty acid oxidation. Wei et al. also described the arrest of cell cycle and induction of apoptosis in human pancreatic cancer cell line (Panc-28) by ROS-mediated mitochondrial depolarization and lysosomal membrane permeabilization.[54] Apoptosis was also induced in several cancer cell lines, including multidrug resistance cancer cells, non-small-cell lung cancer cell lines, lung adenocarcinoma, B16F10 melanoma cells, breast cancer, and colon cancer by oleanolic acid. This compound activates caspase-3, decreases the expression of Bcl-2 antiapoptotic gene, and increases the expression of pro-apoptotic protein Bax. Along with this, oleanolic acid is capable of decreasing angiogenic VEGF and decreasing the development of melanoma-induced lung metastasis of the B16F10 melanoma model in vivo.[7,47,48,55−58] In osteosarcoma cells, oleanolic acid inhibits proliferation and colony formation, induces G1 arrest, and promotes apoptosis, through mTOR signaling, a central regulator of cell growth, proliferation, survival, and metabolism.[59]

Maslinic Acid. Recent studies report the chemopreventive potential of maslinic acid in colorectal cancer in vitro[46] and in vivo.[60] This compound has not been as thoroughly studied as oleanolic acid in cancer, but there is increasing interest in the preventive action that it seems to possess. Hsum et al. studied the chemopreventive action that maslinic acid showed in Raji cells. It suppressed COX-2 expression and inhibited NF-κB and AP-1 binding activities.[61] Targeting pro-inflammatory pathways by dietary phytochemicals as a strategy for cancer prevention is one of the current issues studied, but at a later stage, inflammation and triterpene action will also be discussed.

One aspect of cancer development is the metastatic potential of the tumor. Many authors have recently studied the antimetastatic activity of maslinic acid in DU145 human prostate cancer cells and its mediation via hypoxia-inducible factor-1α signaling (HIF-1α).[62] In these cancer cells, maslinic acid acts by inhibiting uPAR, E-cadherin, VEGF, and matrix metalloproteases (MMPs) expression and dramatically reduces the levels of HIF-1α. Consequently, maslinic acid inhibits the migration, invasion, and adhesion of DU145 prostate cancer cells. As oleanolic, this acid induces apoptosis in specific cancer cell lines.[63−67] In some of them, maslinic acid promotes apoptosis by a mechanism similar to the one of oleanolic acid: a JNK-p53-dependent mechanism, the mitochondrial apoptotic pathway, the increase of expression of Bid and Bax, repression of Bcl-2, release of cytochrome *c*, and increase in caspase-9, -3, and -7 expression. Another potential antitumor activity of maslinic acid is its enhancement of the antitumor activity of TNF-α by suppressing NF-κB action and downstream gene expression, apart from activating the caspase-dependent apoptotic pathway.[68]

Uvaol and Erythrodiol. The two dialcohols of olive oil have been targeted for research in recent years. It was in 1976 when uvaol was first described to possess tumor inhibitory effects, along with ursolic acid and betulinic acid.[69] Until 1994, there were not any additional studies on the effects of any of these compounds. Then, Es-Saady et al. described uvaol, ursolic acid, and oleanolic acid inhibition in leukemic cell line proliferation.[70]

Erythrodiol effects on skin tumor formation in mice were described in 1988,[71] and until 2008, no author had described its cytotoxic effect.[72] Since then, several works have described uvaol and erythrodiol antitumoral effects in murine and human cancer cell lines.[7,73−76] The most remarkable effect of both is their pro-apoptotic potential, which they exert in two different ways: associated with ROS and by c-Jun N-terminal kinase JNK activation.[7,74,76] Again, it seems clear that ROS are crucial in the mechanism of action of these four compounds.

Inflammation, Oxidative Stress, and Oxidative Damage to DNA. Inflammation is related to several diseases, for example, as a prelude for cancer development and interrelating different kinds of cells for the development of a response to a trauma or strange antigen.

Virgin olive oil triterpenes have been recently studied for the modulation that they exert in the inflammatory response.

Oleanolic acid has been described as an anti-inflammatory molecule in vivo[77−79] and in vitro.[80,81] This compound promotes an anti-inflammatory status inhibiting the activation of nuclear factor-κB (NF-κB) and the production of tumor necrosis factor-α (TNF-α) in human umbilical vein endothelial cells (HUVECs).[82] The suppressive effect of triterpenes in the activation of NF-κB seems to be extensive to the four triterpenes in different types of cells.[61,80,81,83] It has been described that the efficient activation of NF-κB-dependent genes by TNF-α requires a cell to be in an oxidized redox state, suggesting that stimuli such as TNF may exert only a limited response if the cell is not in an appropriate redox equilibrium;[84] thus, the link between ROS generation and activation of the NF-κB pathway seems to be recognizable.[85] Most studies focus on the role that triterpenes

could play against certain diseases, their apoptotic role against tumor cells, or the protective action in vascular alteration, but a principal feature of these compounds is their antioxidant effect (Figure 2).

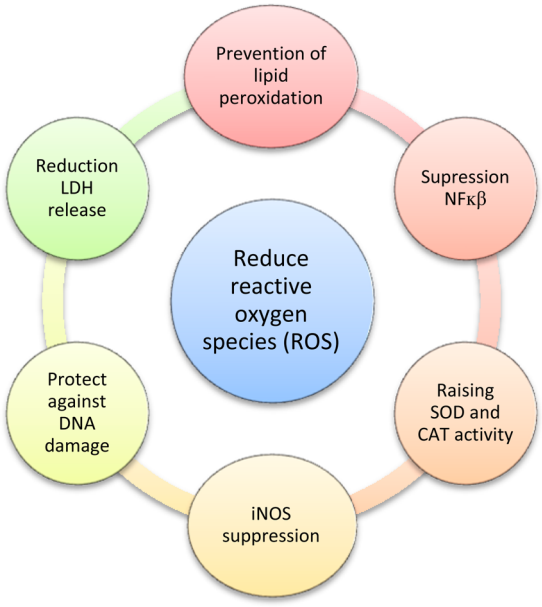

Figure 2. Oxidative mechanisms affected by triterpenes.

The chemical antioxidant role of the triterpenic fraction of virgin olive oil is well documented, although the free radical scavenging activity is almost absent in oleanolic acid, uvaol, and erythrodiol. Maslinic acid also exhibited a weak antiradical activity up to 800 μM and 2.50 mol ratio, but up to 5.00 mol a high DPPH scavenging activity was observed.[7] It acts as an efficient peroxyl radical scavenger by the ORAC assay.[6]

Balanehru et al. described the protection offered by oleanolic acid, isolated from *Eugenia jumbolana*, against hepatic microsome lipid peroxidation in rats.[86] Maslinic acid was described to prevent hepatocyte membrane from lipid peroxidation in rats, induced by the hydroxyl radical (OH*).[87] According to this, some authors tried to study this prevention of lipid peroxidation in hepatic microsomes of rats that were fed, for 3 weeks, high-oleic-acid oils (of sunflower oil, olive oil, and olive pomace oil) containing different concentrations of the antioxidants α-tocopherol, erythrodiol, and oleanolic acid. They concluded that oleanolic acid and erythrodiol protect against, at least partly, microsomal lipid peroxidation in rats fed olive pomace oil.[88]

Oxidative stress and inflammation are closely related, not only because of the NF-κB pathway but also on account of other signals such as ROS and reactive nitrogen species (RNS) produced by macrophages and other mediated immune cells. With this signal, macrophages activate other immune cells that, with them, will try to mediate inflammation and revert to the initial health status. In this way, any compound that acts directly or indirectly in oxidative stress will act in inflammation and, thereby, in the prelude of several diseases.

According to this, oleanolic acid has been one of the triterpenes most studied in inflammation and oxidative stress. This compound is an effective inhibitor of cyclo-oxygenase (COX) and of 5-lipoxygenase (5-LOX),[89] both present in the arachidonic acid synthesis pathway. The anti-inflammatory effects of suppressing COX-2 action, like the reduction of several pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, are well-known.

Other studies in PC12 cells show the influence of oleanolic acid in reducing subsequent H_2O_2- or MMP^+-induced cell death and lactate dehydrogenase (LDH) release, which leads to alleviated oxidative stress in PC12 cells H_2O_2- or MMP^+-induced injury. It spares GSH, raising the activity of SOD and catalase and reducing the release of IL-6 and TNF-α.[93,94] Another antioxidant effect of oleanolic acid was studied by Tsai et al.[95] The study was undertaken in mouse brain, where, dose-dependently, oleanolic acid diminished ROS and proteins related with oxidative stress, showing neuroprotective effects in vivo.

COX-2 and inducible nitric oxide synthethase (iNOS) expression are suppressed at protein and mRNA levels by maslinic acid, and likewise in the translocation of NF-κB to the nucleus (and IκBα phosphorylation), in a concentration-dependent manner in cultured cortical astrocytes.[90] These last actions (reduction of IL-6, IL-1β, and TNF-α) are produced by maslinic acid in mouse macrophages[91] and by the four triterpenic compounds of olive oil in human peripheral blood mononuclear cells.[15] These authors observed that maslinic acid significantly inhibits the enhanced production of NO induced by lipopolysaccharide (LPS), measured by the nitrite production with an IC_{50} value of 25.4 μM. This seems to be in correlation with an action in the iNOS gene expression rather than a direct inhibitory effect on the enzyme activity. ROS were reduced in a dose-dependent manner (IC_{50} = 43.6 μM) showing a preventive effect in oxidative stress in murine macrophages. The inhibition of NO production by oleanolic and maslinic acid was described by Yang et al. in murine RAW 264.7 cells.[92] In breast cancer cells ROS production was decreased by uvaol, oleanolic acid, and maslinic acid.[7]

Interestingly, triterpenes are capable of protecting against H_2O_2-induced DNA damage in several leukemic[96] and human breast cancer cell lines.[7] There are not many studies about antioxidant effects of triterpenes in DNA damage, but attending to the effects observed in different types of cells on oxidative stress, and with these previous studies in leukemic and breast cancer cell lines, probably these triterpenic acids and dialcohols play an important role in the oxidative stress mechanism of the cell, even at nucleus level, protecting against oxidative damage to DNA. Because of that, these olive oil triterpenes could be a good option for preventing different diseases related with oxidative stress, such as cardiovascular diseases,[97] cancer,[98] or even Parkinson's disease[99] and Alzheimer's disease.[100,101]

Another potentially interesting role of triterpenes is their predictable antioxidant capacity in aging. Aging is associated with the accumulation of inactive or less active forms of numerous enzymes. The possibility that these age-related changes are due, at least in part, to oxidative modification is indicated by Berlett et al.[102] There is no scientific evidence of the action of these compounds in the oxidative modification of a protein, but they are modulators of the proteic activity in the cell and could protect against the loss of their activity or oxidative modification; additional studies are required to ensure this.

Oleanolic acid, maslinic acid, uvaol, and erythrodiol are the main triterpenes found in virgin olive oil, but they are not present in other edible oils. They appear in olive leaves and olive skin, and their concentrations depend on the variety selected and the culture handling. These triterpenes possess antioxidant properties per se, and in different cellular types, they affect some central proteins of oxidative stress and inflammation (NF-κB and COX-2); it is still unknown what actual pathways they affect and how.

Although the origins of the diseases described above are varied, oxidative stress is a common condition in them. Compounds that protect against oxidative stress may be useful to prevent these diseases. As we have already showed, triterpenes exert a protective role against oxidant environment, regulating it or, even more, diminishing it. Therefore, the main triterpenes of virgin olive oil could have a critical role in preventing a group of several diseases related with oxidative stress, such as cancer or cardiovascular disease.

More bioavailability studies about these triterpenes are needed to obtain reliable information about the range in which they are present in the cellular metabolism.

Taking into account all available scientific evidence, the beneficial effects of the major triterpenes present in virgin olive oil could prevent certain diseases. For all of these reasons, more studies on the mechanism of action of these triterpenes in oxidative stress are required; indeed, these studies could probe the potential role of triterpenes in preventing the appearance of different diseases.

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Funding

This study was financially supported by the Junta de Andalucía (Proyecto de Excelencia PI10-AGR-6724) and supported by grants from the University of Jaén (Plan de Apoyo a la Investigación-Acción 16).

Notes

The authors declare no competing financial interest.

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Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patients

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- Trabajo enviado a la revista "*Histology and Histopathology*"
- Fecha de publicación: Noviembre de 2013
- Nº de citas (a 1 de Diciembre de 2015): 4
- SCImago Journal & Country Rank
  - Categoría perteneciente: Histology
  - Índice SJR: 0,757
  - H index: 68
  - Posición: 16/57
  - Cuartil en la categoría: Q2
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Pathology
  - Índice de impacto: 2,096
  - Posición: 34/76
  - Cuartil en la categoría: Q2

Histol Histopathol (2013) 28: 1439-1450

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patients

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Summary. Since circulating tumor cells (CTCs) have metastatic potential, their genetic and phenotypic characteristics could provide crucial information to establish the most effective therapy. We assessed the clinical utility of a methodology that allows the simultaneous analysis of CTC phenotype and genotype in colon cancer patients and, in addition, whether this methodology could provide complementary information to that obtained by the primary tumor biopsy.

Thirty-three non-metastatic (stages 0-III) colon cancer patients and 9 healthy donor samples were evaluated. All peripheral blood samples (10 ml) were analyzed by cytokeratin immunomagnetic enrichment. Eight samples were analyzed by immunocytochemistry and 25 samples were analyzed by FICTION technique for simultaneous cytokeratin expression and chromosome 17 and ERBB2 gene status. A further study was carried out in one patient who showed CTC heterogeneity in chromosomal abnormalities. We analyzed HER2 protein expression on CTCs and FISH and HER2 protein expression in primary tumor of this patient.

Our results show that 9.09% of patients had cytokeratin-positive CTCs (CK+/CTCs in peripheral blood). One of the patients showed heterogeneity in chromosomal 17 abnormalities and two different CK expression patterns on CTCs: one CK+/CTCs and one CK-/CTCs. Furthermore, 63.33% of these CTCs overexpressed HER2 protein while the primary tumor of this patient was diploid and did not express HER2 protein.

We describe a methodology that allows the simultaneous genetic and phenotypic analysis of CTCs in colon cancer patients, which may provide essential information to select patients who might benefit from specific therapy.

Key words: CK+/CTCs, Colon cancer, ERBB2, FICTION, HER2

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Introduction

Colon cancer is one of the most common types of tumors in developed countries (Jemal et al., 2011). Despite the improvement of survival rates achieved with the use of new chemotherapy agents, approximately 20% of stage II patients will die from recurrent disease (Le Voyer et al., 2003), which shows the need for more effective therapies.

Different genetic alterations that have been described in colorectal cancer may represent novel candidates for tailored therapy, such as i) alterations in

protooncogene C-MYC (located on chromosome 8) that encodes a transcription factor that is one of the most potent and frequently deregulated oncoproteins in humans cancers (Facchini and Penn, 1998); ii) alterations in the ERBB2 gene (located at chromosome 17), a member of the EGFR family, it is a common target for breast cancer, but recent studies have found overexpression or amplifications in other solid tumors, such as lung, gastric and colorectal cancers (Nathanson et al., 2003; Takenaka et al., 2011; Tsapralis et al., 2012). This offers the possibility of testing ERBB2-targeted drugs, including trastuzumab and lapatinib, in these subpopulations of patients (Kuwada et al., 2004; Kelly et al., 2010; Javle and Hsueh, 2010; Morishita et al., 2010; Choi et al., 2011). Overexpression or amplification of ERBB2 has been reported in 3%-14% of primary tumors from colorectal cancer patients (Dursun et al., 2001; Nathanson et al., 2003; Al-Kuraya et al., 2007; Kavanagh et al., 2009), and some authors have suggested that the addition of trastuzumab to chemotherapy might increase the clinical response in these colon cancer patients (Kuwada et al., 2004; Al-Kuraya et al., 2007; Kavanagh et al., 2009; Kelly et al., 2010; Javle and Hsueh, 2010; Morishita et al., 2010; Choi et al., 2011).

The detection of circulating tumor cells (CTCs) appears to be an important prognostic marker for different tumor types, such as breast cancer, where it serves as a progression marker and indicator of overall survival (Gaforio et al., 2003; Cristofanilli et al., 2004, 2005; Müller et al., 2006). In addition, the detection of CTCs identifies therapy resistant breast cancer patients (Cristofanilli et al., 2005; Camara et al., 2007; Pierga et al., 2008). The prognostic significance of CTCs in colorectal cancer is not clear yet, although recent reports suggest an association with overall survival and disease progression (Koch et al., 2006; Cohen et al., 2008; Sastre et al., 2008). However, little attention has been paid to the cytogenetic features of such cells.

In this study we analyzed CTCs of 33 non-metastatic colon cancer patients with the goal of evaluating ERBB2 status in such CTCs. Our methodology allows us to analyze simultaneously the ERBB2 phenotype and genotype of single cytokeratin positive (CK+)/CTCs isolated from peripheral blood (PB) by immunomagnetic separation and FICTION (Campos et al., 2008). We found that 3 out of 33 patients had detectable CK+/CTCs (9.09%) and 1 of them had CK+/CTCs with strong HER2 expression. CK+/CTCs in this patient were triploid for chromosome 17 and did not show ERBB2 amplification, while the primary tumor showed disomy for this chromosome and ERBB2 gene, and were negative for HER2 protein expression. Therefore we were able to identify CTCs in PB with different genetic and phenotypic characteristics to the ones found in primary tumor biopsy. Since CTCs are linked to tumor recurrence and metastasis, CTCs' genotype and phenotype analysis could also complete the real status of the disease with metastatic and primary tumor analysis.

Materials and methods

Cell lines and controls

The human colon cancer cell lines Caco-2, HT-29, and human breast cell lines MCF-7, MDA-MB-231 were obtained from the American Type Cultured Collection (ATCC; Rockville, MD). The human breast cancer cell line SK-Br-3 (with ERBB2 amplification and overexpression) was obtained from Eucellbank (Barcelona, Spain). MCF-7 and MDA-MB-231 cells were grown in MEM with Earle's salt (PAA Laboratories, Pasching, Austria), supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAA Laboratories, Pasching, Austria) and 1% of a stock solution of penicillin-streptomycin (Sigma, St. Louis, MO). Caco-2, HT-29 and SK-Br-3 cells were grown in RPMI 1640 (PAA Laboratories, Pasching, Austria), plus 10% (v/v) heat-inactivated fetal calf serum and 1% of a stock solution of penicillin-streptomycin. Cells in the exponential growth phase were used for the experiments. These cancer cell lines were used as positive control. Briefly, we spiked 1000 of each tumor cell line in 10 ml of peripheral blood (PB) from 6 healthy donors (one sample of blood per each tumor cell line except for SK-Br-3 control that was duplicated), under an approved ethical protocol and signed informed consent. Previously, we validated the absence of CTCs in PB from healthy donors as negative controls. Besides these healthy donor samples, we also evaluated three PB samples of patients with diverticulosis disease (benign inflammatory disease) as negative controls. Positive controls, negative controls and patient's samples were carried out separately to avoid cross contamination and were processed following our previously published FICTION protocol (Campos et al., 2008).

Study population and clinicopathologic examination

Informed consent was obtained from all participants following an explanation of the nature of the study, as approved by the research ethics board of our hospital. All patients were considered sporadic cases on the basis that no clinical antecedents of familial adenomatous poliposis were reported, and those who met the clinical criteria for hereditary nonpolyposis colon cancer (Amsterdam criteria) were excluded. Between June 2004 and November 2005, blood samples (10 mL) were taken from 33 non-metastatic colon cancer patients (stages 0-III) by venipuncture, the day before surgery. All patients were followed up until death or until August 11, 2007. Follow-up of the patients was carried out every 6 months and included recording of the development of local and distant tumor relapse and the survival state of the patients.

The following variables were obtained from the

medical records of the 33 patients: Age, gender, tumor location, pathologic stage, histological differentiation and type, lymph node metastases, tumor invasion, and evidence of polyps (defined by the presence of polyps in the surgical sample). Pathologic stage was assessed using tumor-node-metastases (TNM) classification (Table 1).

Blood samples, density gradient separation and CK+/CTCs enrichment by immunomagnetic separation

Peripheral venous blood samples (10 mL) were collected from colon cancer patients before surgery. PB samples were collected in heparinized tubes (BD Vacutainer, Becton Dickinson; Heidelgerg, Germany) and were processed within 4 hr after collection. Eight PB samples were processed according to the methodology previously described by our group (Gaforio et al., 2003) and 25 PB samples were processed following our FICTION protocol (Campos et al., 2008).

Briefly, a double-density ficoll gradient was prepared per PB sample (Histopaque 1119 and Histopaque 1077 (Sigma, St. Louis, MO)). After centrifugation, the mononuclear cell fraction and the granulocyte fraction were isolated. Then tumor cells were immunoseparated using the Carcinoma Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), with some modifications. After nucleated cells were isolated, permeabilizated and fixed, the samples were incubated with Fc-Blocking Reagent and immunomagnetically labeled with MACS anti-cytokeratin microbeads (microbeads conjugated to a monoclonal anti-cytokeratin 7/8 antibody; clone: CAM 5.2). After this, samples were placed onto a MACS Column (Miltenyi Biotec, Bergisch Gladbach, Germany) and the retained cells (magnetic-positive cell population; cytokeratin-positive (CK+) cells) were eluted. The magnetically enriched cell fractions were spun down onto poly-L-lysine-coated glass slides (Sigma, St. Louis, MO) in a cytocentrifuge (Hettich; Tuttlingen, Germany). Then slides were air-dried over-night at room temperature and stored at -20°C without fixation.

Detection of CK+/CTCs by immunocytochemistry

Eight PB samples were processed by immunocytochemistry. After thawing, slides were stained with anti-cytokeratin 7/8 conjugated to FITC (isotype: mouse IgG2a. Miltenyi Biotec, Bergisch Gladbach, Germany) and further labeled with anti-FITC antibody conjugated to alkaline phosphatase (isotype: mouse IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany). Then samples were incubated with Fast Red TR/Naphthol AS-MX substrate solution (Miltenyi Biotec, Bergisch Gladbach, Germany), counterstained with Mayer's hematoxylin, and mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) following our immunocytochemistry protocol (Gaforio et al., 2003). CK+/CTCs were counted separately by two expert researchers.

Detection of CK+/CTCs by FICTION (Fluorescence immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasm)

Twenty five PB samples were processed by FICTION (Campos et al., 2008). Briefly, after thawing and fixation, these samples were incubated with a blocking solution (10% rabbit serum (Sigma, St. Louis, MO)) followed by primary antibody incubation with the biclonal mouse anti-AE1-AE3 antibody (BioGenex, San

Table 1. Correlation among CK+/CTCs and clinical/morphological variables.

Clinical or pathological variables	Number of CK+/CTCs/10 ml		p value
	CK+ patients	CK- patients	(%)
Gender			
Male	2 (6.06%)	20 (60.60%)	0.718
Female	1 (3.03%)	10 (30.30%)	
Tumor location			
Ascending colon	2 (6.06%)	12 (36.36%)	0.477
Transverse colon	0 (0.00%)	1 (3.03%)	
Descending colon	0 (0.00%)	12 (36.36%)	
Sigmoid colon	1 (3.03%)	4 (12.12%)	
Transverse-sigmoid colon	0 (0.00%)	1 (3.03%)	
Grade of differentiation			
Well	2 (6.06%)	10 (30.30%)	0.289
Moderate and moderate-poor	0 (0.00%)	13 (39.39%)	
Poor	1 (3.03%)	5 (15.15%)	
TNM			
0	0 (0.00%)	2 (6.06%)	1.000
I	0 (0.00%)	3 (9.09%)	
II	1 (3.03%)	10 (30.30%)	
III	2 (6.06%)	15 (45.45%)	
Histological type			
Adenocarcinoma	3 (9.09%)	26 (78.78%)	1.000
Mucinous adenocarcinoma	0 (0.00%)	2 (6.06%)	
Intramucosal adenocarcinoma	0 (0.00%)	2 (6.06%)	
Tumor configuration			
Excrescent	0 (0.00%)	6 (18.18%)	0.501
Excrescent-infiltrative	0 (0.00%)	1 (3.03%)	
Infiltrative	1 (3.03%)	7 (21.21%)	
Polyploid	0 (0.00%)	5 (15.15%)	
Ulcerative	1 (3.03%)	1 (3.03%)	
Ulcer vegetative	1 (3.03%)	9 (27.27%)	
Vegetative	0 (0.00%)	1 (3.03%)	
Tumor invasion			
Lymphatic			
+	2 (6.06%)	15 (45.45%)	0.523
-	1 (3.03%)	15 (45.45%)	
Venous			
+	0 (0.00%)	3 (9.09%)	0.744
-	3 (9.09%)	27 (81.81%)	
Perineural (n=10)			
+	1 (3.03%)	8 (24.24%)	0.629
-	2 (6.06%)	22 (66.67%)	

Ramon, CA). The development was conducted by applying 2 sequential layers of secondary antibodies: Alexa Fluor 350 rabbit anti-mouse IgG and goat anti-rabbit IgG (Invitrogen, Eugene, OR). After fluorescent immunophenotyping, slides were evaluated under a Leica TCS-DL confocal laser scanning system with argon and two helium neon lasers on a Leica DM IRB inverted microscope, with appropriate filter set. Blue fluorescent images were acquired with Leica DFC 300FX digital camera and processed with a Leica IM50 image manager. Confocal images were acquired and processed using Leica confocal LCS software (Leica Microsystems; Wetzlar, Germany).

After assessment of positive tumor cells on the slides, samples were fixed and dehydrated in series of ethanol (Panreac Quimica, Barcelona, Spain). Afterwards, samples were codenatured (85°C for 5 min) and hybridized (overnight at 37°C) with the LSI HER2/CEP17 multi-color probe, (ERBB2 (HER2/neu) probe labeled with SpectrumGreen and a chromosome enumeration probe CEP17 labeled with SpectrumAqua) (Abbott Molecular, Vysis; Des Plaines, IL). Posthybridization wash was performed at 72°C in 2xSodium chloride citrate buffer (SCC) (MP Biomedicals Europe, Illkirch, France)/0.3% Igepal (Sigma, St. Louis, MO) for 2 min, followed by another wash at room temperature. Microscopic evaluation was carried out with the Leica microscope described above by two expert researchers.

Analysis of hybridization

All CK+/CTCs were scored to determine the number of hybridization signals for each ERBB2 and CEP17 probe. The absolute copy numbers and the relative copy number ratio (ratio between the absolute number of ERBB2 signals in each CK+/CTC and the absolute number of their own chromosome 17 centromere signals) were determined. For FISH evaluation a [ERBB2]/CEP17 ratio value >1.5 was considered to be an increase copy number of the gene. Similarly, ratio value ≤0.7 was considered to be a decrease copy number or the gene, and ratios between 1.3 and 1.5 were considered as equivocal values, and were interpreted with caution.

Statistical analysis

The patient characteristics were related to the presence of CK+/CTCs in PB by using Fisher's exact test. When a variable was ordered a trend analysis was applied. Actuarial curves for progression-free survival (PFS) and overall survival (OS) were calculated by the Kaplan-Meier method. PFS and OS were calculated from the date of the primary detection of CK+ cells in PB to the date of disease progression or death. Disease progression was defined as metastatic recurrence of non-metastatic patients.

Study of patient with identification number (IN) 28

Due to the genetic characteristics of the CK+/CTCs found in IN 28 patient, we conducted a further study of this patient by assessing several tumor markers, genetic characteristic of the primary tumor and the expression of HER2 protein in CK+/CTCs of this patient.

Immunohistochemistry of primary tumor

Immunostaining of primary tumor sample was assessed by Department of Pathology (University Hospital of Jaén) on routinely processed paraffin sections using the citrate buffer (pH 6) (Master Diagnostica, Granada, Spain) as an antigen-retrieval method. Sections were examined with monoclonal antibodies (mAbs) recognizing p53 (Novocastra, Newcastle upon Tyne, UK); cyclin D1 (Dako, Glostrup, Denmark); HER2 (Dako, Glostrup, Denmark); Ki-67 (Concepta Biosystems, Barcelona, Spain); CD31 (Novocastra, Newcastle upon Tyne, UK); and CD34 (Novocastra, Newcastle upon Tyne, UK). Immunohistochemical staining was performed with a sensitive polymer-based system (Dako EnVision system, Glostrup, Denmark) with diaminobenzidine (DAB) solution as a chromogen (Dako, Glostrup, Denmark). All incubations were carried out at room temperature. The sample was counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany) and mounted using standard procedures. Positive and negative (substituting the primary antibody by distilled water) controls were included in the kit. All recommended prognostic factors by the American College of Pathologists were recorded.

The assessment of immunohistochemistry was conducted using an Olympus BH-2 microscope (Olympus; Hertfordshire, United Kingdom) attached to a Nikon Coolpix 5400 camera for acquisition of digital images. All markers were quantitated using random fields that were recorded as digital images under high power view (x400) and counting a mean of 1000 cells per marker with the aid of the ImageJ software for image analysis (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007).

FISH of primary tumor

The ERBB2 evaluation by FISH was performed on two sections (3 μm) from paraffin-embedded tissue sample. Briefly, after paraffin was removed the sections were rehydrated in ethanol series and treated with hydrochloric acid (Panreac Quimica, Barcelona, Spain), followed by a treatment with 8% Sodium thiocyanate (Panreac Quimica, Barcelona, Spain) and digested with 0.025% pepsin (Sigma, St. Louis, MO) in hydrochloric acid. Samples were then rinsed with 2xSSC, dehydrated in ethanol series and air dried at room temperature.

Next, dual-color probe Poseidon™ Repeat Free™

ERBB2, HER2/Neu (17q12) & SE 17 Control probe (red color for ERBB2, and green for SE17) (Kreatech Diagnostics, Amsterdam, The Netherlands) were added to slides and codenaturation (75°C for 10 min), hybridization (overnight at 37°C) and posthybridization wash (72°C in 1xSSC for 2 min, followed by 1xSSC at room temperature) were performed following the Kreatech Diagnostic's recommended protocol. Slides were mounted with DAPI I counterstain (Abbott Molecular, Vysis; Des Plaines, IL).

Evaluation of the hybridization was done with a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss; Jena, Germany), with the appropriate filters set. ISIS software (MetaSystems; Altslussheim, Germany) was used to capture the images. 100 tumor nuclei were examined and alterations of the number of signals were evaluated following standard procedures.

Immunostaining of CK+/CTCs with anti-HER2 mAb (HercepTest, Dako)

After FICTION evaluation, IN 28 sample was dismounted, rehydrated and the anti-HER2 antibody was applied as described in the section Immunohistochemistry of primary tumor. Positive controls, in which FICTION had been evaluated, were included in the immunostaining of CK+/CTCs with anti-HER2, in order to have a control of the immunocytochemistry. These controls followed the same process as described for the IN28 sample, except for one of the SK-Br-3 controls that was used as a negative control (substituting the primary antibody with distilled water to assure that crossed reactions or false positive results did not occur).

Analysis of HER2 expression

Tumor cell lines and CTCs from IN 28 sample were scored according to manufacturer's guidelines. Score 0 was defined as no staining of tumor cells, score 1+, as faint membrane staining of these cells; score 2+, as weak to moderate membrane staining; and a score of 3+, as strong staining of the entire membrane with perinuclear reinforcement. Samples were considered negative when a score 0 or 1+ staining were found in >10% of tumor cells, while they were considered positive when score 2+ or 3+ was observed in >10% of these cells. Microscopic evaluation was carried out with the Leica microscope described above.

Results

Detection of CK+/CTCs in colon cancer patients

Blood samples from 33 consecutive patients with colon cancer were taken to measure CK+/CTCs levels, and no statistical association was found between presence of CK+/CTCs and any of the variables studied (Table 1). The negative controls, PB samples from three patients with diverticulosis disease and 6 healthy volunteers were negative for CK+ cells detection.

CK+/CTCs were detected in 3 out of 33 PB samples (9.09%). The mean number of CK+/CTCs isolated in these 3 patients was 9.67 (range 1-26 CK+ cells), relationship between the presence of CK+/CTCs and OS or/and PFS was not found (median follow-up = 13 months; range = 0.5-29.9).

Regarding the three CK+/CTCs patients, one of them was analyzed by immunocytochemistry and showed one CK+/CTC with a strong cytoplasmic staining pattern, whereas the surrounding hematopoietic cells showed no CK expression (data not shown). The genetic characteristic of this CK+/CTC could not been studied due to technical problems. The other two samples were analyzed by FICTION and results are show in the section FICTION *analysis on the immunoselected CK+/CTCs*.

Characterization of tumor cells lines by FICTION and HER2 expression

We first established the characterization of the FICTION technique, using tumor cell lines spiked in blood samples from healthy volunteers.

Carcinoma cell lines in positive controls were unequivocally distinguished among the white blood cells by their CK blue-labelling (Fig. 1). Hybridization signals for ERBB2 and CEP17 were also clearly observed in both tumor and white blood cells (Fig. 1). All leukocytes showed two signals for the ERBB2 gene and centromere 17, thus serving as internal controls for the

Table 2. Chromosomal 17 aneusomy according to HER2 protein expression scoring on CK+/CTCs from IN 28 patient.

Chromosomal aneusomy	Number of CK+/CTCs	Percentage of CK+/CTCs		HER2 protein expression score					Total CK+/CTCs
				0	1+	2+	3+	2+/3+	
Trisomy	23	88.46%	Number of CK+/CTCs	0	8	5	6	11	19
			Percentage of CK+/CTCs	0.00%	42.11%	26.32%	31.58%	57.89%	
Disomy	3	11.54%	Number of CK+/CTCs	0	0	2	0	2	2
			Percentage of CK+/CTCs	0.00%	0.00%	100.00%	0,00%	100.00%	
Total CK+/CTCs	26		Number of CK+/CTCs	0	8	7	6	13	21
			Percentage of CK+/CTCs	0.00%	38.09%	33.33%	28.57%	61.90%	

hybridization.

The five positive controls (SK-Br-3, Caco-2, HT-29, MCF-7 and MDA-MB-231) and SK-Br-3 negative control (in which we substituted the primary antibody for distilled water), were analyzed for the immunostaining with anti-HER2 protein. Different expression patterns were found on each positive control (Fig. 1). SK-Br-3, Caco-2 and HT-29 tumor cells showed a strong staining (3+ score). MCF-7 tumor cells displayed a moderate staining (annotated as 2+) and MDA-MB-231 cells were negative for HER2 protein expression.

FICTION analysis on the immunoselected CK+/CTCs

Criteria for CK+/CTCs identification consisted of nucleated cells with a malignant phenotype (Fig. 2 "Transmission column" shows a representative example of morphology of isolated CK+/CTCs), staining positive

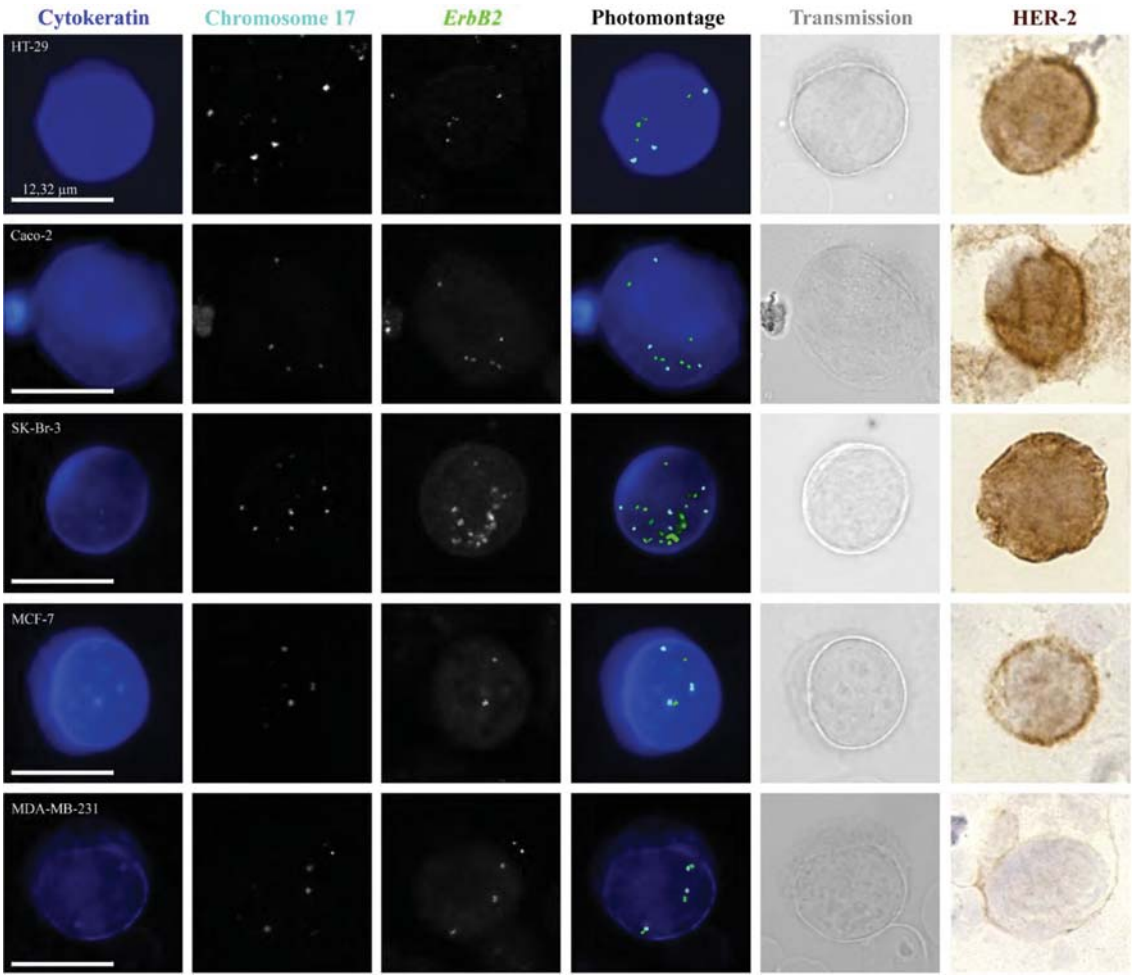

Fig. 1. Fluorescent images (x100 objective) of PB from healthy volunteers spiked with different tumor cell lines [HT-29, Caco-2, SK-Br-3, MCF-7 and MDA-MB-231] processed by immunomagnetic enrichment and FICTION techniques. Column "Cytokeratin" displays the CK labelling of tumor cells which are clearly identified as blue fluorescent, evaluation by fluorescence microscopy. Columns "Chromosome 17" and "ErbB2" show the hybridization signals for CEP17 and ERBB2 (HER2/neu) respectively (overlay of different layers), evaluated by confocal microscopy. Column "Photomontage" displays the overlay of Cytokeratin (blue), Chromosome 17 (aqua or light blue) and ERBB2 (green). Column "Transmission" shows the morphology of tumor cells, analyzed with confocal microscopy. The expression of HER2 protein is shown in the last column "HER-2". These cells are different from the ones photographed in the other columns. Scale bar: 12.32 μm

for cytokeratin (CK+ cells) in blue fluorescence. Results were expressed as number of CK+/CTCs per 10 mL PB. In addition, to ensure the efficiency of hybridization, a minimum of 50 morphologically intact and non-overlapping nuclei of normal cells (contaminated leukocytes) were scored per sample and used as internal control.

IN 21 Patient: A total of 2 intact cells were detected as CK+/CTCs in this patient. These CK+/CTCs were evaluated for the hybridization of ERBB2 gene signals. Both CK+/CTCs presented disomy for this chromosome and showed 2 copies for ERBB2 gene (data not shown).

IN 28 Patient: A total of 26 intact CK+/CTCs with a malignant phenotype were found in this patient. Among the 26 CK+/CTCs studied, 23 CK+/CTCs showed polysomy (trisomy) for chromosome 17 (88.46%) and 3 CK+/CTCs presented disomy for this chromosome (11.54%) (Table 2). The CEP17 average number of copies for these CK+/CTCs was 2.88 copies per cell. 20 CK+/CTCs (out of these 26 CK+/CTCs) were evaluated in terms of absolute copy number of ERBB2 signals. Four CK+/CTCs (4/20) presented 3 copies for this gene (20.00%) and 16 (16/20) showed 2 copies for ERBB2 gene (80.00%). In summary, amplification of ERBB2 gene was not detected. The ERBB2 gene status for these CK+/CTCs was 2.20 copies per cell. Figure 2, "ERBB2 and Chromosome 17 columns" shows a representative example of ERBB2 gene and chromosome 17 status of isolated CK+/CTCs.

HER2 protein expression on CTCs

Evaluation of HER2 protein expression was only possible in 21 of the 26 CK+/CTCs. HER2

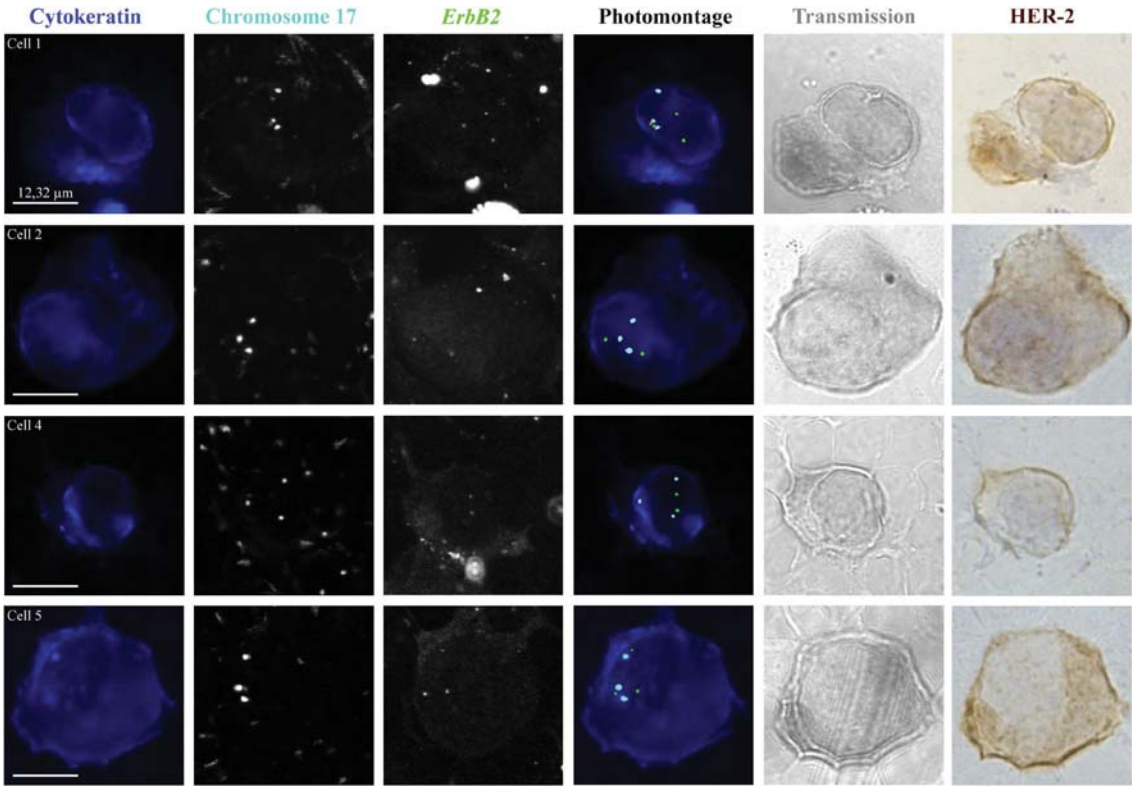

Fig. 2. Fluorescent images (x100 objective) of CK+/CTCs isolated from PB from IN 28 patient. Column "Cytokeratin" displays the CK labelling of these cells, which are clearly identified by their blue fluorescence. Samples were analyzed by fluorescence microscopy. Columns "Chromosome 17" and "ErbB2" show the hybridization signals for CEP17 and ERBB2 (HER2/neu) respectively (overlay of different layers, confocal microscopy). Column "Photomontage" displays the overlay of Cytokeratin (blue), Chromosome 17 (aqua or light blue) and ERBB2 (green). Column "Transmission" shows the morphology of CK+ cells (confocal microscopy). The expression of HER2 protein is shown in the last column "HER-2". All CK+/CTCs are the same as those photographed in the other columns. Scale bar: 12.32 μm

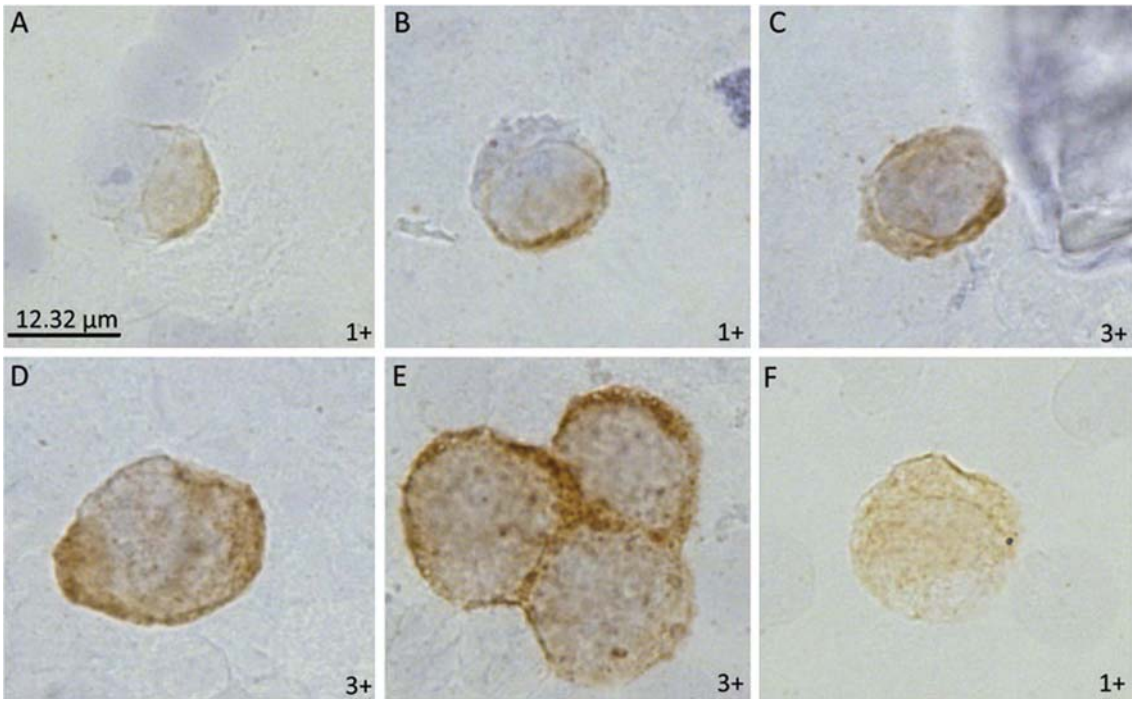

Fig. 3. HER2 expression on CK-/CTCs (x100 objective) from IN 28 patient. The score of HER2 protein expression is shown in the lower right corner of the images. Scale bar: 12.32 μm.

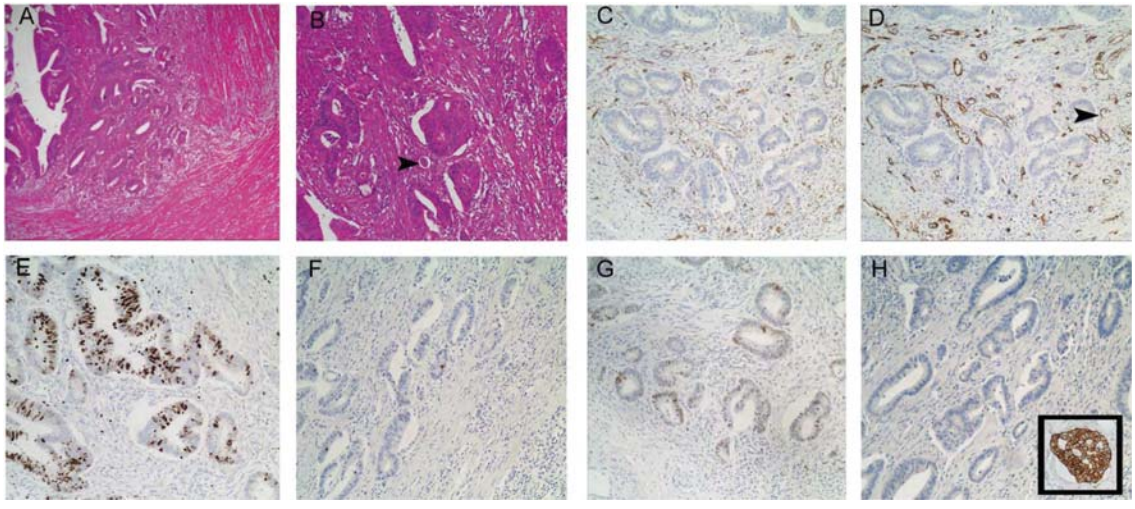

Fig. 4. Histopathological features of the tumor from IN 28 patient (stage II). **A.** Neoplastic glands with infiltrative pattern of growth affecting the muscular propria layer of the bowel. Haematoxylin-eosin. **B.** Detail from the neoplastic glands, atypical but well conformed, although fused glands are present. Note the presence of lymphovascular (small vessel) invasion (arrowhead). Haematoxylin-eosin. **C-G.** Immunohistochemical expression of the markers in the tumor, showing high microvessel density as seen by CD31 **(C)** and CD34 **(D)** immunostaining. The arrowhead corresponds to small vessel invasion. High proliferative index (Ki-67) **(E)**, low p53 expression **(F)**, high expression of cyclin D1 **(G)** and Herceptest **(H)** negative test define the molecular characterization of this tumor (insert into the lower right quadrant of figure: Positive control from intraductal breast carcinoma). All figures: Envision® method. A, x 10 objective; B, x 40 objective; C-K, x 20 objective.

overexpression was detected in 13 of 21 CK+/CTCs (61.90%); 6 CK+/CTCs showed a 3+ staining and 7 CK+/CTCs presented a 2+ staining. Eight CK+/CTCs (38.10%) displayed a faint membrane staining and were scored as negative-expression. Finally, 5 out of these 26 CK+/CTCs were not evaluable. Figure 2 "HER2 column" shows a representative example of HER2 expression on the isolated CK+/CTCs.

In addition, we found 9 tumor cells that were CK negative (CK-)/CTCs, but histopathological examination revealed that they were tumor cells. In these 9 CK-/CTCs, HER2 overexpression was detected in 6 of them, with a 3+ staining; and 3 tumor cells showed faint membrane staining (scored as negative for HER2 expression) (Fig. 3). In summary, 19 (13 CK+/CTCs and 6 CK-/CTCs) CTCs had HER2 overexpression (63.33%) and 11 (8 CK+/CTCs and 3 CK-/CTCs) CTCs were negative for HER2 (36.67%).

Immunohistochemistry and FISH of the primary tumor tissue for IN 28 patient

Focusing on IN 28 patient (stage II) we decided to make a deeper study of the primary tumor. Histological examination revealed an infiltrative adenocarcinoma (Fig. 4) without positive nodes or distant metastases (he was followed up until April 15, 2008). Mayer's haematoxylin & eosin-stained slides showed a high grade of tumor aggressiveness (Fig. 4A; B). The tumor stained strongly for Ki-67 (up to 90% of stained cells) (Fig. 4E), cyclin D1 (about 50%) (Fig. 4G), with nearly nil expression of p53 (Fig. 4F) and no expression of HER2 (Fig. 4H). Immunohistochemical demonstration of CD31 and CD34 showed well-developed microvasculature as well as the presence of tumour cells within small vessels (Fig. 4C; D respectively).

FISH analysis of two different sections demonstrated no ERBB2 amplification and disomic pattern for chromosome 17 (data not shown). The negative expression of HER2 is in line with the FISH result found in the primary tumor, but neither of them matched with the CK+/CTCs' results of this patient.

Discussion

In this study, we analyzed genetic alterations of chromosome 17 and ERBB2 gene by FICTION in CK+/CTCs of 25 PB samples from a total of 33 non-metastatic colon cancer patients (stages 0-III). Some studies in breast cancer have demonstrated that HER2 expression can be acquired in CTCs from HER2 negative tumor during tumor progression (Meng et al., 2004; Fehm et al., 2007, 2010), and that detection of these CTCs with overexpression of HER2 could be associated with a worse prognosis (Wülfing et al., 2006). Therefore, we decided to study the genetic status of this gene in our series of patients. We have also chosen the analysis of ERBB2/HER2 protein because there is a monoclonal antibody against this protein that is nowadays being used in breast cancer treatment (Slamon et al., 2001; Madarnas et al., 2008). Furthermore, this treatment could be used in other types of tumors with ERBB2 amplifications or HER2 protein overexpression (Langer et al., 2004; Javle and Hsueh, 2010) and in patients with ERBB2-amplified or HER2+ CTCs (Meng et al., 2004).

It should be noted that few studies have been focused on the genetic characteristics of CTCs in cancer patients. Only two reports have applied cytogenetic techniques in search of chromosome aneuploidies in of CTCs from PB in metastatic (Fehm et al., 2002) and non-metastatic colon cancer patients (Wind et al., 2009). One of them indicates that CTCs were heterogeneous for chromosomal abnormalities and the gain of chromosomes was more frequent than the genetic loss (Fehm et al., 2002). These results are in agreement with findings in other tumors types, such as breast (Fehm et al., 2002) or prostate cancer (Fehm et al., 2002; Swennenhuis et al., 2009). Few studies have applied FISH technique to evaluate specific genes status in CTCs such as the concordance of ERBB2 amplification in CTCs and primary tumors in breast cancer patients (Meng et al., 2004); or the amplification of androgen receptor (AR) and MYC genes in CTCs from prostate cancer patients (Swennenhuis et al., 2009).

In this article, we have demonstrated the utility of a method that combines an immunomagnetic selection of CTCs from peripheral blood of colon cancer patients with FICTION technique. FICTION technique was developed by Weber-Matthiesen et al. (1992) to assign tumor cells a cytogenetically defined clone and to determine their specific cell lineage at one time. FICTION technique allows genetic and phenotypic CTC characterization without cell membrane destruction and subsequent cell relocalization, which typically is done in FISH, which the subsequent time consuming and loss of evaluable tumor cells (Swennenhuis et al., 2009). Furthermore, we demonstrate the feasibility to study second marker expression by immunocytochemistry on the same CTC.

The most interesting finding in this work was, apart from demonstrating the validity of the technique to analyze simultaneously CK+/CTCs' phenotype and genotype, the discovery of a gain in chromosome 17 and HER2 overexpression in a patient with HER2 negative primary tumor. FISH analysis of the primary tumour demonstrated disomy for chromosome 17 and ERBB2 gene. A total of 26 CK+/CTCs were isolated from the PB of this colon cancer patient. These tumor cells were an heterogeneous cell population with basically two patterns. The predominant one (~88.00%) showed trisomy of chromosome 17 and the second pattern was characterized by disomy for this chromosome (~11.00%). Both populations of these CK+/CTCs presented 2 copies of ERBB2 gene (~80%) but neither of them had ERBB2 amplifications. In contrast, when we analyzed the expression of HER2 protein, 61.90% of the CK+/CTCs showed overexpression (63.33% if we

also counted CK-/CTCs). This patient was scored as HER2+ CTCs (more than 10% of CTCs were HER2+), and some studies showed that evaluation of only 10 CTCs could indicate the patient's HER2 status (Meng et al., 2004, 2006; Cao et al., 2010).

Our finding of the existence of a heterogeneous CTC population in the same PB sample, and the tumor cells CK expression variability are in keeping with the results shown in metastatic colon (Fehm et al., 2002), prostate and breast cancer patients (Mikolajczyk et al., 2011). The fact that CTCs in this patient overexpressed HER2, whereas the primary tumor did not, can be explained by the possibility that these CTCs may come from an unusual subclone of tumor cells that are not readily detectable by primary tumor biopsy. Alternatively, CTCs could express HER2 *de novo*, since this may confer a survival advantage to metastatize. Obviously, we cannot conclude that colon cancer patients with HER2 overexpression in CTCs should be treated with trastuzumab (Herceptin) therapy. However, it is worth noticing that breast cancer patients with ERBB2-amplified CTCs were treated with trastuzumab and showed a partial or complete remission (Meng et al., 2004). Another few studies hypothesize that HER2 CTC status determination could be a tumoral marker for the use of HER2-targeted therapies (Fehm et al., 2007, 2010). Future studies would be needed to determine the convenience of targeting ERBB2/HER2 in colon cancer.

We have only detected 3 CK+/CTC in a total of 33 (9.09%) non-metastatic colon cancer patients. In addition, CK+/CTCs detection did not correlate with primary tumor characteristics or with PFS or OS. This percentage of CK+/CTC-positive patients is lower than those published in previous studies (Cohen et al., 2008; Sastre et al., 2008; Maestro et al., 2009). However, other studies have reported similar percentages of CTCs in PB to those shown in our study (Wind et al., 2009; Thorsteinsson et al., 2011). The main difference between these two sets of studies is the study population, which may explain the disparity found between these results.

In metastatic colorectal cancer patients (Cohen et al., 2008, 2009; Molnar et al., 2001), where the sample collection was taken postoperatively and before chemotherapy treatment was initiated, a number of CTCs higher than 2 or 3 correlated with shorter OS and/or PFS (Cohen et al., 2009; Maestro et al., 2009) and only the presence of this tumor cells identified chemotherapy resistant patients (Molnar et al., 2001).

In non-metastatic colon cancer patients, analysis of CTCs in peripheral blood before surgery or chemotherapy treatment rendered a similar percentage to the one found here. Wind et al. (2009) tested the detection of CTCs in PB and portal blood in different sets of samples in 31 non-metastatic colon cancer patients at different time points: before tumor mobilization, after tumor mobilization and post-surgery. This report showed a CTC detection rate of 4-7% in peripheral blood vs. 26-54% in portal blood, depending on when blood samples were obtained, and described no correlation between tumor cell detection and clinicopathologic characteristics or disease progression. Thorsteinsson et al. (2011) found that 5% of samples (1/20 non-metastatic colon cancer patients) before surgery were positive for CTCs whereas samples taken after surgery were negative. This study reported no correlation between CTC detection and the tumor characteristics, PFS or OS. Therefore, our results suggest that basal levels of CTC (or CK+/CTCs) in non-metastatic colon cancer patients may have no prognostic value, although further studies with larger series of patients should be carried out to address this issue.

Cohen et al. (2006) theorized that colorectal cancer biology could play an important role in CTCs' recovery, since this cancer disseminated more frequently via portal blood than other epithelial tumors, which disseminated via peripheral blood, such as breast and prostate cancer. Thus, these authors suggested that metastatic colorectal cancer patients might be less likely to have CTCs in peripheral blood than metastatic breast or prostate cancer patients. Consequently, in non-metastatic patients it would be more noted. For example, Wind et al. (2009) detected a higher percentage of non-metastatic colon cancer patients with CTCs in portal than in peripheral blood (54%; 31%; 45% in portal blood vs. 7%; 4%; 4% in peripheral blood, these samples were taken before tumor mobilization, after tumor mobilization or post-surgery, respectively).

In the patient where we found gain of chromosome and HER2 overexpression in CTCs, we may speculate that this would confer metastatic advantage. In breast cancer, it has been previously reported that HER2 overexpression can be acquired during tumor progression: CTCs may express HER2 although their primary tumor was considered as HER2 negative (Meng et al., 2004; Fehm et al., 2007, 2010). The detection of these CTCs with HER2 overexpression could be associated with a worse prognosis (Wülfing et al., 2006). Although the use of HER2-targeting drugs (such as trastuzumab) is not yet approved for colon cancer, clinical trials are underway to test its efficacy in the selected small population of HER2+ patients.

The CTCs are traditionally considered as one of the sources of the metastatic disease's origin (Pantel and Brakenhoff, 2004; Paterlini-Brechot and Benali, 2007). Additionally, genetic changes can occur at any stage of tumor progression (Meng et al., 2004, 2006). Thus, we hypothesize that the CTCs analysis would be useful to know the real status of the disease and, in the future, could be considered as a routine analysis in cancer patients.

Conclusions

The conclusions from our study are as follows: (i) individualized CTC analysis using the technique described in the present article may provide additional information to conventional pathological diagnosis; (ii) chromosome 17 triploidy and HER2 overexpression can

appear in CTCs of early stage colon cancer patients.

Acknowledgements. This study was supported by the "Fundación de Investigación Médica Mutua Madrileña" (to J.J. Gaforio).

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Accepted May 6, 2013

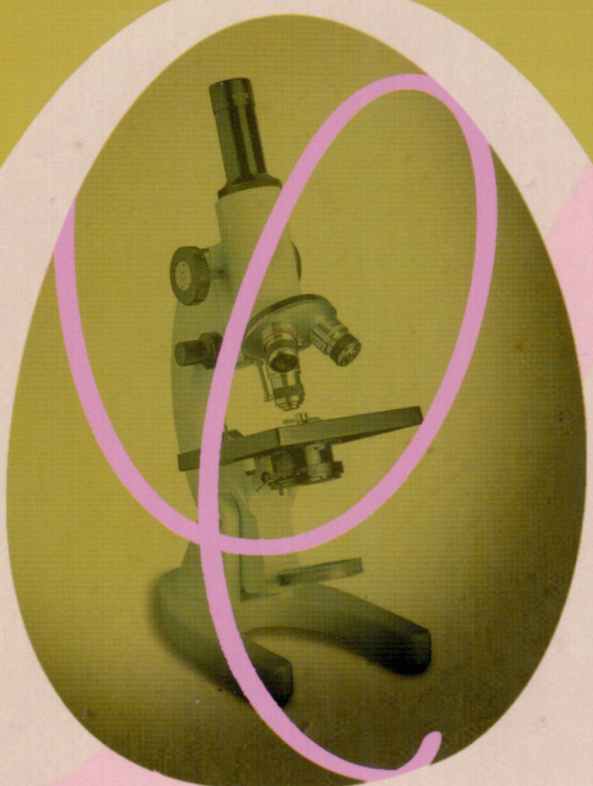
Influencia de los Componentes Minoritarios Presentes en los Aceites de Oliva Vírgenes en la Prevención del Cáncer de Mama. Estudio in vitro del Hidroxitirosol y Tirosol

José Juan Gaforio, Fernando Warleta y Cristina Sánchez-Quesada

- Editorial: Graficas la Paz, Torredonjimeno (Jaén).
- Editado por: Castillo de Canena Olive Juice
- Fecha de publicación: Mayo de 2012
- Páginas: 77
- Idioma: Español
- ISBN: 978-84-695-3347-5

Influencia de los componentes minoritarios presentes en los **aceites de oliva vírgenes** en la prevención del **cáncer de mama**

Estudio **in vitro** del **hidroxitirosol y tirosol**



José J. *Gaforio* | Fernando *Warleta* | Cristina *Sánchez-Quesada*

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Universidad de Jaén

CASTILLO DE CANENA
tradición olivarera desde 1780

El presente trabajo resultó ganador del I Pemio Castillo de Canena
de Investigación Oleícola "Luis Vañó" fallado el 14 de Noviembre de 2011
por un Jurado compuesto por los siguientes miembros:
D. Manuel Parras Rosa, Presidente.
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D. Leopoldo Martínez Nieto
Doña Milagros Saavedra Saavedra
D. Sebastián Sánchez Villasclaras
Doña Mariela Chova Martínez

La presente edición de 500 ejemplares ha sido financiada en su totalidad
por Castillo de Canena Olive Juice como parte de su política
de responsabilidad social corporativa.

EDICION NO VENAL

Primera edición, abril 2012

Depósito Legal: J-698-2012
ISBN:

Imprime: Gráficas la Paz de Torredonjimeno
www. graficaslapaz. com

ÍNDICE

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Allouche Y., Warleta F., Campos M., Sánchez-Quesada C., Uceda M., Beltrán G., Gaforio J.J.

- Trabajo enviado a la revista *"Journal of Agricultural and Food Chemistry"*
- Fecha de publicación: Enero de 2011
- Nº de citas (a 1 de Diciembre de 2015): 52
- SCImago Journal & Country Rank
  - Categoría perteneciente: Agricultural and Biological Sciences
  - Índice SJR: 1,148
  - H index: 199
  - Posición: 23/204
  - Cuartil en la categoría: Q1
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Food Sciences and Technology
  - Índice de impacto: 2,912
  - Posición: 13/123
  - Cuartil en la categoría: Q1

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 121–130 121
DOI:10.1021/jf102319y

Antioxidant, Antiproliferative, and Pro-apoptotic Capacities of Pentacyclic Triterpenes Found in the Skin of Olives on MCF-7 Human Breast Cancer Cells and Their Effects on DNA Damage

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This research aimed to investigate erythrodiol, uvaol, oleanolic acid, and maslinic acid scavenging capacities and their effects on cytotoxicity, cell proliferation, cell cycle, apoptosis, reactive oxygen species (ROS) level, and oxidative DNA damage on human MCF-7 breast cancer cell line. The results showed that erythrodiol, uvaol, and oleanolic acid have a significant cytotoxic effect and inhibit proliferation in a dose- and time-dependent manner. At 100 μM, erythrodiol growth inhibition occurred through apoptosis, with the observation of important ROS production and DNA damage, whereas uvaol and oleanolic acid growth inhibition involved cell cycle arrest. Moreover, although all tested triterpenes did not show free radical scavenging activity using ABTS and DPPH assays, they protected against oxidative DNA damage at the concentration 10 μM. Uvaol and oleanolic and maslinic acids, tested at 10 and 100 μM, also reduced intracellular ROS level and prevented H_2O_2-induced oxidative injury. Overall, the results suggest that tested triterpenes may have the potential to provide significant natural defense against human breast cancer.

KEYWORDS: Pentacyclic triterpenes; *Olea europaea*; olive oil; human breast cancer; antiproliferative activity; cell cycle arrest; apoptosis; antioxidant activity; DNA damage

INTRODUCTION

Breast cancer is the leading cause of mortality in women in developing countries (*1*). Of all environmental factors known to influence breast cancer, diet appears to be one of the most significant (*2*). Table olives and olive oil constitute regular dietary components of the traditional Mediterranean diet, which has been associated with a low incidence and prevalence of certain types of cancers, including breast cancer (*3*, *4*). This healthy property is mainly ascribed to oleic acid (*5*), phenolic compounds (*6*), and squalene (*7*,*8*). Nonetheless, other minor components have showed relevant interesting activities. Among them, erythrodiol, uvaol, oleanolic acid, and maslinic acid (**Figure 1**) are the main pentacyclic triterpenes located in the skin of olive fruits (*9*). These constituents are present in virgin olive oil (*10*) with higher concentration in olive pomace oil (*11*, *12*). Among other biological activities, including anti-inflammatory and cardioprotective (*13*−*15*), these triterpenes were reported to possess antioxidant and antitumor properties. Indeed, they were shown to prevent lipid peroxidation (*16*, *17*), protect low-density lipoproteins (LDL) against oxidation (*15*, *18*), and suppress superoxide anion generation (*19*, *20*). Furthermore, these bioactive compounds were found to inhibit the growth of tumor cell lines from various human cancers (*21*−*25*). Despite these numerous papers, to the authors' best knowledge, to date there are no available studies regarding the effects of these triterpenes on breast cancer cells. Therefore, the present study was undertaken to investigate the antioxidant capacity of erythrodiol, uvaol, oleanolic acid, and maslinic acid and its relationship to their antiproliferative capacity and oxidative DNA damage protection, using MCF-7 cells as a model for malignant breast cancer cells. For this purpose, we studied triterpenes' scavenging activity and their effects on cell growth, cell cycle profile, apoptosis, intracellular oxidative stress, and DNA oxidative damage.

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MATERIALS AND METHODS

Chemicals and Reagents. The following reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO): Hepes buffer; sodium pyruvate; nonessential amino acids mixture 100× (NEAA); 2′,7′-dichlorofluorescein diacetate (DCFH-DA); 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT sodium salt); 5-methylphenazinium methyl sulfate, *N*-methylphenazonium methyl sulfate (PMS); DL-*all-rac*-α-tocopherol (vitamin E); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 2,2-diphenyl-1-picylhydrazyl (DPPH); 2,2′-azinobis(3-ethylbenzthiazolie-6-sulfonic acid) diammonium salt tablets (ABTS); phosphate buffer saline (PBS); and Hank's buffered salt solution (HBSS). Minimum essential medium with Eagle's salts (MEM), fetal bovine serum (FBS), and phenol-red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express and propidium iodide (PI) were

Published on Web 12/10/2010

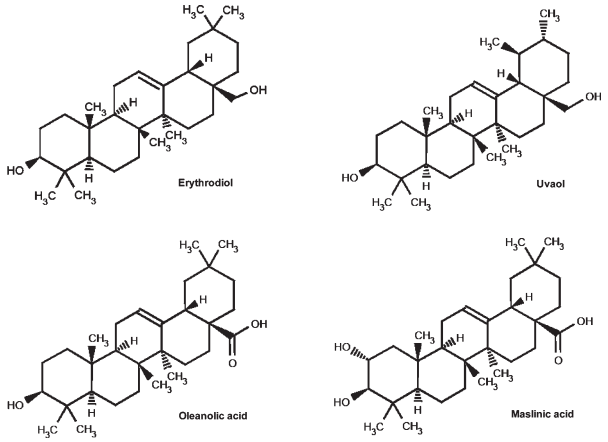

Figure 1. Chemical structures of erythrodiol, uvaol, oleanolic acid, and maslinic acid.

obtained from Invitrogen (Eugene, OR). $K_2S_2O_8$ was obtained from Panreac Quimica S.A.U (Barcelona, Spain). Culture plates were obtained from NUNC (Roskilde, Denmark). The PI/RNase Staining Buffer kit, FITC-conjugated annexin V, and binding buffer were obtained from BD Biosciences Pharmigen (San Diego, CA.). The comet assay kit was purchased from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD).

Erythrodiol, uvaol, and oleanolic acid (purity ≥ 97, 98.5, and 99%, respectively) were purchased from Extrasynthese (Genay, France). Maslinic acid (purity > 80%) was provided by Dr. A. Garcia-Granados, Department of Organic Chemistry, University of Granada, Spain, and was obtained according to the patented method (*26*). Stock solutions of these compounds were prepared in ethanol and frozen at −20 °C until use. For cell experiments, these stock solutions were then diluted in MEM to reach the desired concentration. The final concentration of ethanol in the medium was < 0.6% for the highest concentration tested and had no significant effect on cell viability. Controls also received the same amount of ethanol in all experiments.

ABTS Radical Scavenging Assay. ABTS cation radical scavenging activity was determined using a previously reported procedure (*27*). ABTS radicals ($ABTS^{\bullet+}$) were obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{\bullet+}$ was diluted in ultrapure water until the absorbance at 734 nm was 0.7 (±0.1). Stock solutions of tested triterpenes and Trolox (as antioxidant reference) in ethanol (10 mM) were diluted with ultrapure water to reach the assayed concentrations. Triterpene concentrations ranged from 12.5 to 800 μM, whereas Trolox concentrations were from 50 to 800 μM. Twenty microliters of each concentration of triterpene, standard (Trolox), blank (ultrapure water), or ethanol control (8%) was added to a 96-well plate. The reaction was initiated by the addition of 280 μL of $ABTS^{\bullet+}$. Absorbance readings were taken every 5 min at 30 °C during 120 min in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate. The inhibition of $ABTS^{\bullet+}$ was calculated according to the following percentage of free radical scavenging activity (% RSA) formula:

$$\%\ \mathrm{RSA} = [(A_{C(0)} - A_{A(t)})/A_{C(0)} \times 100]$$

Where $A_{C(0)}$ is the absorbance of the control (blank) at $t = 0$ and $A_{A(t)}$ is the absorbance in the presence of the triterpene or standard sample at $t = 60$ min.

DPPH Free Radical Scavenging Assay. The antioxidant activity of the tested triterpenes against the stable radical DPPH was measured as previously reported by Brand-Williams et al. (*28*) with some modifications. Briefly, a 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of triterpenes in 96-well plates at 0.13, 0.25, 0.5, 1, 2.5, 5, and 10 mol of antioxidant/mol of DPPH. α-Tocopherol was used as a standard antioxidant control in the range of concentration 0.13−1.00 mol ratio. A sample without antioxidant was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min during 120 min in a microplate reader (TECAN GENios plus). Measurements were performed in triplicate. The inhibition of DPPH radical was calculated according to the percentage of free radical scavenging activity (% RSA) described above (at $t = 60$ min).

Cell Lines and Culture. The MCF-7 (primary human breast cancer) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and was maintained at 37 °C in a humidified atmosphere under 5% CO_2 in MEM supplemented with 10% FBS, 1% Hepes buffer, 1% sodium pyruvate, and 1% NEAA. Cells in the exponential growth phase were used for all experiments.

MDA-MB-231 (metastatic human breast cancer) and U937 (human histiocytic lymphoma) cell lines were used to compare the effect of triterpenes on apoptosis induction. These cell lines were cultivated using the same MCF-7 culture conditions.

Cytotoxicity Assay. The effect of the tested triterpenes on cell viability was determined by the XTT assay, which is based on the ability of live cells to cleave the tetrazolium ring, thus producing formazan, which absorbs at 570 nm (*29*). A total of 5×10^3/well MCF-7 cells were grown onto 96-well plates for 24 h prior to treatment with increasing concentrations of triterpenes, from 12.5 to 100 μM, for another 24 h. Thereafter, plates were incubated with XTT in RPMI without phenol red for 3 h, and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). All measurements were performed in triplicate, and each experiment was repeated at least three times.

Cell Proliferation Assay. In the proliferation assay, MCF-7 cells were seeded at a density of 2×10^3/well onto 96-well culture plates and allowed to adhere for 24 h. Thereafter, medium was replaced with fresh medium containing increasing concentrations of tested triterpenes, from 12.5 to 100 μM, and plates were incubated for 24, 48, or 120 h. After each time point, medium was substituted by a fresh culture medium and cells were allowed a proliferation period of 120, 96, or 24 h, respectively. Then, plates were incubated with XTT in RPMI without phenol red for 3 h, and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus) (*8*). All measurements were done in triplicate, and each assay was made twice.

Cell Cycle Assay. MCF-7 cells were seeded in 12-well culture plates at a density of 1×10^5/well. After 24 h, the cells were treated with or without (control) 10 and 100 μM triterpenes under study and incubated for an additional 24 h. They were then fixed with cold 70% ethanol and stored at −20 °C for at least 24 h. Cells were subjected to flow cytometry analysis on an EPICS XL-MCL cytofluorometer (Beckman Coulter, Spain) after propidium iodide labeling (PI/RNase staining buffer). The percentage of cells in sub G1, G0/G1, S, and G2/M phases was calculated using the FlowJo program (v5.7.2). The experiment was repeated at least three independent times.

Apoptosis Assay. The percentage of apoptotic cells was determined using a double staining assay with FITC-conjugated annexin V and propidium iodide (PI). MCF-7 cells (1×10^5/well) in 12-well culture plates were treated after 24 h with or without 10 and 100 μM triterpenes for another 24 h. Subsequently, cells were harvested, washed twice in cold PBS, and resuspended in 100 μL of annexin binding buffer. Cells were stained with 5 μL of annexin V−FITC and 1 μL of PI solution, gently vortexed, and incubated for 15 min at room temperature in the dark before flow cytometric analysis. The experiment was repeated at least three independent times.

Detection of Intracellular Reactive Oxygen Species. The generation of intracellular reactive oxygen species (ROS) was evaluated on the basis of the intracellular peroxide-dependent oxidation of DCFH-DA to form a fluorescent compound, DCF, as described by Warleta et al. (*8*). Briefly, MCF-7 cells were seeded at a density of 1×10^4/well on 96-well plates and grown for 24 h to allow adhesion. Subsequently, the medium was substituted with a fresh one containing 1, 10, or 100 μM tested triterpenes, and cells were incubated for another 24 h. Thereafter, cells were washed two times with HBSS and incubated with fresh DCFH-DA (100 μM) for 30 min at 37 °C in 5% CO_2. Cells were then washed twice and read in a plate reader for 30 min (Ex λ_{485}/Em λ_{535}, gain 60). The intracellular ROS level percentage was calculated as

$$F = [(F_{t=30} - F_{t=0})/F_{t=0} \times 100]$$

where $F_{t=0}$ is the fluorescence at $t = 0$ min and $F_{t=30}$ the fluorescence at $t = 30$ min.

Hydrogen peroxide (H_2O_2), a widely used model of in vitro oxidative stress, can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell (*30*). To evaluate whether erythrodiol, uvaol, oleanolic acid, or maslinic acid is able to reduce induced oxidative

Table 1. Percentage of Free Radical Scavenging Activity of Erythrodiol, Uvaol, Oleanolic Acid and Maslinic Acid Measured by Decolorization of $ABTS^{\bullet+}$ (**A**) and Reduction of the DPPH Radical (**B**)[a]

(**A**)

	Trolox	erythrodiol	uvaol	oleanolic acid	maslinic acid
12.5 μM	ne	3.49 ± 0.34	4.08 ± 0.55	3.69 ± 0.43	4.39 ± 0.36
25 μM	ne	2.61 ± 0.39	3.59 ± 0.36	3.19 ± 0.39	3.91 ± 0.24
50 μM	17.61 ± 0.38	2.74 ± 0.26	3.57 ± 0.63	3.19 ± 0.43	4.83 ± 0.49
100 μM	32.77 ± 0.77	2.30 ± 0.41	5.02 ± 0.86	2.66 ± 0.66	4.34 ± 0.37
200 μM	58.70 ± 1.32	2.83 ± 0.56	5.25 ± 1.04	3.62 ± 1.09	5.86 ± 0.46
400 μM	94.99 ± 0.77	2.08 ± 0.64	4.37 ± 1.05	5.92 ± 1.76	10.03 ± 0.68
800 μM	99.06 ± 0.24	2.28 ± 0.16	4.60 ± 1.36	5.60 ± 1.67	18.32 ± 0.86

(**B**)

mol AH/mol/DPPH	α-tocopherol	erythrodiol	uvaol	oleanolic acid	maslinic acid
0.13	44.33 ± 1.71	—	2.86 ± 0.63	—	0.20 ± 0.07
0.25	71.64 ± 2.10	—	1.69 ± 0.65	—	0.33 ± 0.11
0.50	81.50 ± 1.25	—	1.46 ± 0.61	—	0.75 ± 0.22
1.00	81.24 ± 1.19	—	2.26 ± 0.57	0.60 ± 0.35	2.08 ± 0.11
2.50	ne	3.47 ± 1.14	4.40 ± 0.64	3.42 ± 0.29	24.45 ± 0.23
5.00	ne	3.35 ± 0.69	3.42 ± 0.69	2.61 ± 0.61	42.37 ± 0.44
10.00	ne	8.24 ± 0.63	12.51 ± 1.19	10.48 ± 1.08	71.93 ± 0.38

[a] Trolox and α-tocopherol were used as standard antioxidants control. Values represent the mean ± SEM of three independent experiments at t = 60 min. ne, not estimated. —, free radical scavenging capacity was not detected.

stress, cells were challenged with 500 μM H_2O_2 30 min before fluorescence quantification.

All measurements were performed in triplicate, and each experiment was repeated at least three times.

Alkaline Single-Cell Gel Electrophoresis (Comet Assay). MCF-7 cells were seeded at the density of 1×10^5/well into 12-well plate cultures. After 24 h, they were treated with or without 10 and 100 μM triterpenes for an additional 24 h. They were then scraped, washed twice (300*g*, 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free), and resuspended in 1 mL of cold 1× PBS. After that, the comet assay was performed according to the method of Warleta et al. (*8*).

To assess whether triterpenes are able to protect against oxidative DNA damage, cell suspensions were exposed for 10 min to 50 μM H_2O_2 at 4 °C. Cells washed twice with cold 1× PBS and resuspended in 1 mL of cold 1× PBS were then subjected to the comet assay procedure.

Slide Scoring and Analysis. DNA strand breaks were examined in a fluorescence microscope (Zeiss Axiovert 200) equipped with a Luca EMCCD camera (Andor Technology, Belfast, U.K.) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, U.K.). Fifty cell images were randomly characterized per sample using 20× magnification. Relative fluorescence between head and tail through the Olive tail moment (Olive_TM) was used to determine DNA damage.

Statistical Analysis. The results of free radical scavenging activity of tested triterpenes (% RSA) are presented as the mean of three independent experiments ± the standard error of the mean (SEM).

For cell assays, data are displayed as the mean of at least three independent experiments ± SEM, and results are expressed as a percentage relative to the untreated control cells, which was defined as 100%. A general variance analysis (ANOVA) was carried out on all data followed by Fisher's LSD test. A p value of < 0.05 was considered to be statistically significant. These statistical analyses were performed using Statgraphics Plus 5.1 statistical software (Statpoint Technologies, Inc., Warranton, VA).

RESULTS

Antioxidant Activity. The free radical scavenging capacity of triterpenes under study was evaluated by means of ABTS and DPPH assays. As shown in **Table 1**, erythrodiol, uvaol, and oleanolic acid have shown a weak free radical scavenging activity in both tests. Maslinic acid also exhibited a weak antiradical activity up to 800 μM and 2.50 mol ratio; however, from 5.00 mol ratio, a high DPPH scavenging activity was observed (RSA > 70% at 10.00 mol ratio).

Effects of Triterpenes on Cell Survival. MCF-7 cells were exposed to increasing concentrations, from 12.5 to 100 μM, of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then cell survival, compared with untreated controls, was evaluated using the XTT assay. As shown in **Figure 2**, the percentage of living cells decreased in a dose-dependent manner, respectively, from 25 and 50 μM erythrodiol and uvaol. It is worth noting that at the same concentration of 100 μM, the cytotoxic effect induced by erythrodiol was significantly stronger than that induced by uvaol (respectively, 12 and 64% of the cells still viable). Related to triterpenic acids, cell survival dropped slightly by 50 or 100 μM oleanolic acid (89% of the cells still viable), whereas it was unaffected by the presence of maslinic acid.

Effects of Triterpenes on Cell Proliferation. The effects of tested triterpenes on MCF-7 cell proliferation was evaluated with concentrations ranging from 12.5 to 100 μM after 24, 48, and 120 h of exposure (**Figure 3**). A dose- and time-dependent decrease in cell proliferation rate was achieved by erythrodiol and oleanolic acid. Cell proliferation was almost completely inhibited 24 h after MCF-7 exposure to 50 μM erythrodiol and 100 μM oleanolic acid (**Figure 3A**,**C**). Uvaol, however, showed a biphasic behavior; an increase in proliferation rate was observed at the concentration of 12.5 μM, followed by a dose- and time-dependent proliferation inhibition from 25 μM (**Figure 3B**). Finally, maslinic acid increased the MCF-7 cell proliferation rate (**Figure 3D**).

Effects of Triterpenes on Cell Cycle and Apoptosis. Figure 4 shows the effects of triterpenes on the different phases of the cell cycle. MCF-7 treatment with 10 μM erythrodiol induced a significant increase in the population in G0/G1 phase (8%), whereas a 100 μM concentration of this compound caused a marked decrease in the percentage of cells in G0/G1 (33%) and an increase in the percentage of cells in sub G1 phase (10-fold higher than untreated control cells) (**Figure 4A**). This sub G1 is normally associated with apoptosis. When cells were treated with a 10 μM

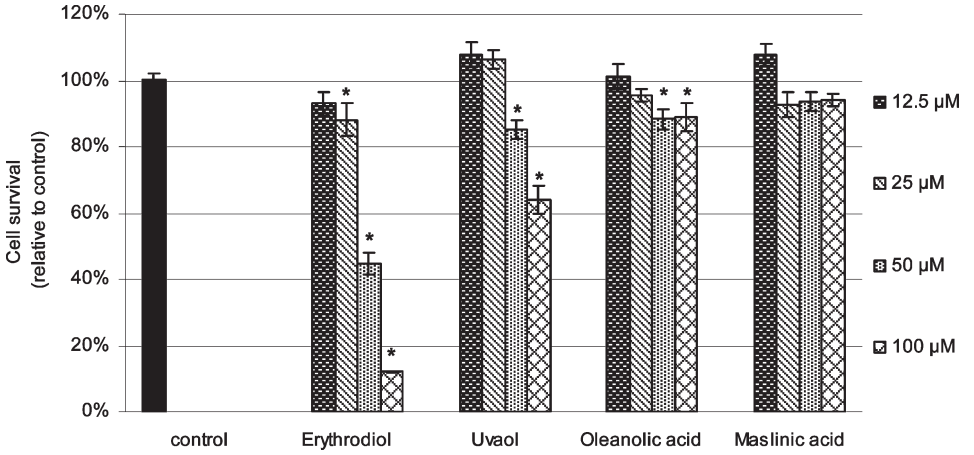

Figure 2. Effects of triterpenes on MCF-7 cell survival. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then cell survival was determined by the XTT assay. The results represent the mean ± SEM of three independent experiments. * denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$).

concentration of either uvaol or oleanolic acid, no changes on cell cycle profiles were detected with respect to untreated controls. At the concentration of 100 μM, uvaol induced a significant increase in G0/G1 phase (8%) and a decrease in S phase (41%) (**Figure 4B**). Similarly, 100 μM oleanolic acid caused an increase in the population in G0/G1 (18%), with a concomitant decrease in the percentage of cells in the S phase (50%) and G2/M phase (34%) (**Figure 4C**). At both concentrations tested, maslinic acid did not induce significant change in the cell cycle profile (**Figure 4D**).

Assessment of apoptosis was performed by flow cytometry using annexin V−FITC and PI labeling, and the percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells. Consistent with cell cycle analysis, 100 μM erythrodiol strongly induced apoptosis (the apoptotic rate increased from 12% in control untreated cells to 64%), whereas no effect on apoptosis induction was observed by the rest of the triterpenes (**Figure 5A**).

In the same way, 100 μM erythrodiol strongly induced apoptosis in MDA-MB-231 and U937 cells (the apoptotic rate increased from 7 to 83% and from 1 to 80%, respectively). The rest of the triterpenes did not induce apoptosis in MDA-MB-231, but they did in U937 cells (**Figure 5B**).

Effects of Triterpenes on Intracellular ROS Level. Measurement of intracellular ROS level using the DCFH-DA probe showed a significant decrease in ROS level in MCF-7 cells after 24 h of treatment with uvaol, oleanolic acid, and maslinic acid. However, in the case of erythrodiol, a marked increase in intracellular ROS level was observed at the concentration of 100 μM, whereas no significant effect was observed at a 1 or 10 μM concentration of this triterpene (**Figure 6A**).

To induce intracellular oxidative stress, H_2O_2 was added before fluorescence measurement. As shown in **Figure 6B**, H_2O_2 induced oxidative injury on MCF-7 cells. Thereafter, to investigate the in vitro preventive effect of triterpenes against H_2O_2 oxidative injury, the intracellular ROS level was measured in MCF-7 cells previously treated with 1, 10, and 100 μM triterpenes. Our results revealed that uvaol, oleanolic acid, and maslinic acid protected against oxidative injury as compared to H_2O_2 control cells in the range of concentrations tested, whereas, as expected, 100 μM erythrodiol did not show any protective effect, showing again an important increase in intracellular ROS level.

Effects of Triterpenes on DNA Integrity and H_2O_2-Induced DNA Damage. The effects of triterpenes under study on DNA integrity and H_2O_2-induced DNA damage in MCF-7 cells was evaluated using alkaline single-cell gel electrophoresis (comet assay), and results were expressed as Olive_TM. Olive_TM incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail) (*31*). Results showed that neither uvaol, oleanolic acid, or maslinic acid, at both doses tested, nor erythrodiol, at the lowest dose, affected DNA integrity. However, significant DNA damage was produced by 100 μM erythrodiol as compared with control (**Figure 7A**).

MCF-7 treatment with 50 μM H_2O_2 alone for 10 min (control H_2O_2) significantly increased Olive_TM when compared with control (**Figure 7B**). Preincubation of MCF-7 cells with a 10 μM concentration of either erythrodiol, uvaol, or oleanolic acid exerted a preventive effect against H_2O_2-induced DNA damage (respectively, 63, 43, and 26% reduction), whereas at 100 μM no longer was a preventive effect observed. Otherwise, MCF-7 cells treated with maslinic acid significantly decreased the formation of single-strand breaks (up to 76% reduction at 100 μM) (**Figure 7B**).

DISCUSSION

In the present study, we have focused on erythrodiol, uvaol, oleanolic acid, and maslinic acid, four pentacyclic triterpenes found in *Olea europaea* (*9*−*12*). Previous papers have described the antiproliferative properties of these triterpenes against various cancer cells (*21*−*25*). However, their antitumor capacity against breast cancer has not yet been studied. Here, we report on the antiproliferative and antioxidant capacities of these triterpenes in the MCF-7 human breast cancer cell line, the first such study to date. Our results suggest that tested triterpenes may have the potential to provide significant natural defense against human breast cancer. This conclusion is based on the following findings: antiproliferative and cytotoxic effects, cell cycle arrest and/or induction of apoptosis, reduction of intracellular ROS level, and prevention against oxidative DNA damage, all depending on dose and chemical structure.

Cytotoxicity, a common preliminary method, is helpful to determine whether tested compounds have potential antineoplastic properties (*32*). Furthermore, increased proliferation and decreased cell death (apoptosis) are two major processes that contribute to the progression of tumor cell growth. The cytotoxic effect of oleanolic and maslinic acids has been previously reported on several tumor cell lines such as A549 (non-small-cell lung cells), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF-498 (central nervous system), HCT-15 (colon), HSC-2 (oral squamous cell carcinoma), and

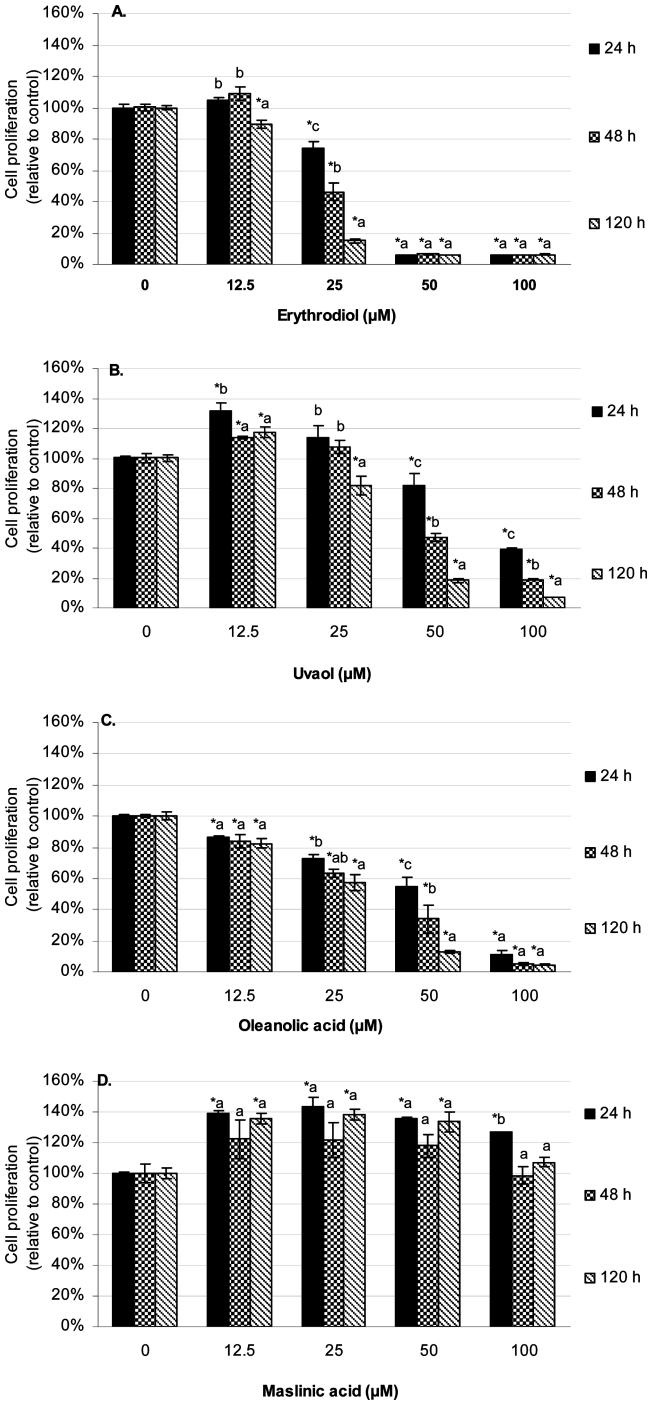

Figure 3. Effects of triterpenes on MCF-7 cell proliferation. Cells were treated with different concentrations of erythrodiol (**A**), uvaol (**B**), oleanolic acid (**C**), and maslinic acid (**D**) for 24, 48, and 120 h, and then cell proliferation, using the XTT assay, was determined after a proliferation period of up to 6 days. The results represent the mean ± SEM of two independent experiments and are expressed as percentage of the control, which was defined as 100%. ∗ denotes statistically significant difference between concentrations for each time ($p < 0.05$). Different letters denote statistically significant difference between times for each concentration ($p < 0.05$).

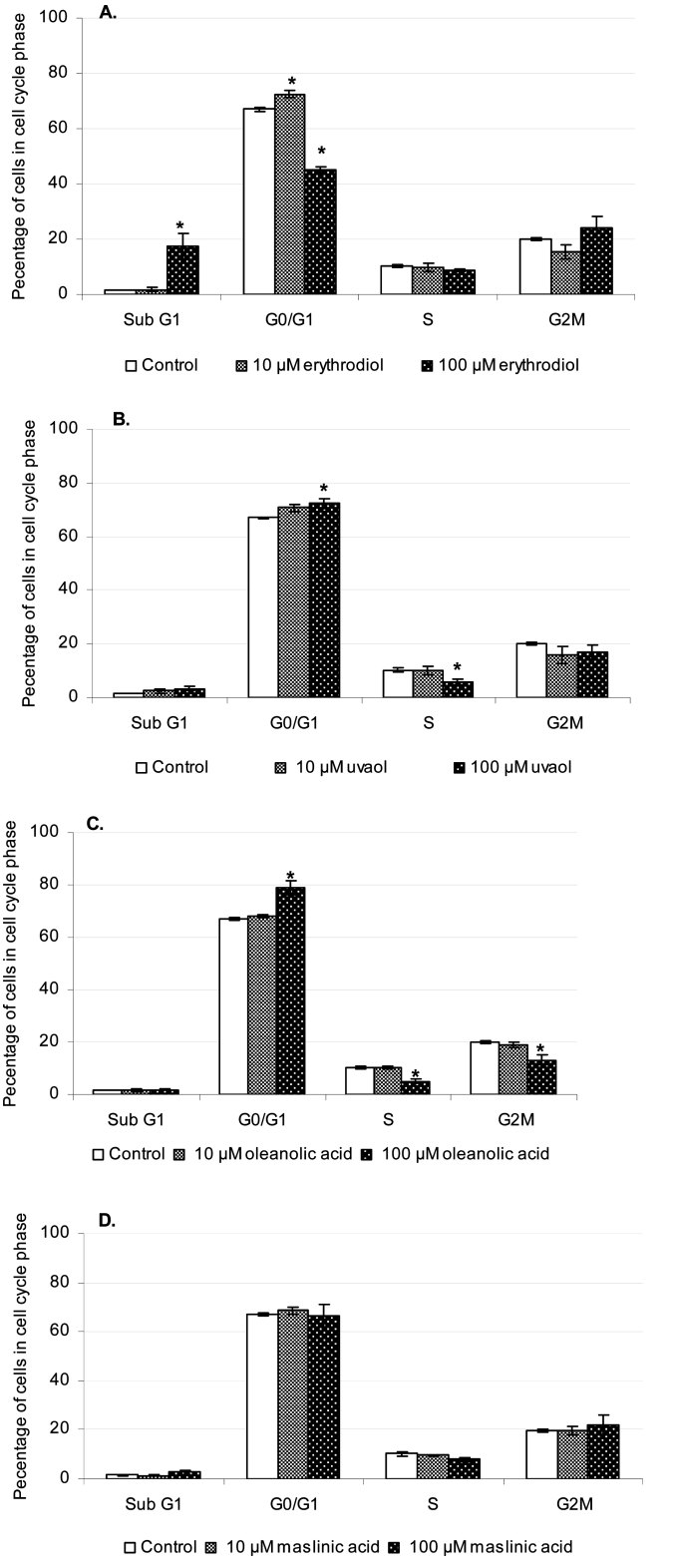

Figure 4. Effects of triterpenes on MCF-7 cell cycle distribution. Cells treated with 10 and 100 μM erythrodiol (**A**), uvaol (**B**), oleanolic acid (**C**), and maslinic acid (**D**) for 24 h were collected and stained with PI after fixation by 70% ethanol. Following flow cytometry, cell cycle distribution was analyzed using the FlowJo program. The results represent the mean ± SEM of four independent experiments. ∗ denotes statistically significant difference compared with control ($p < 0.05$).

HSG (salivary gland tumor) (*33*, *34*). In addition, the antiproliferative capacity of erythrodiol, uvaol, oleanolic, and maslinic acids was described in Caco-2 and HT-29 colon cancer cell lines (*21*−*23*) and in 1321N1 astrocytoma cell line (*24*, *25*). In this study, our results showed that erythrodiol, uvaol, and oleanolic acid significantly inhibited cell growth and proliferation in a dose- and time-dependent manner, the effect of erythrodiol being more pronounced. Hence, these data suggest that these triterpenes effectively reduced the malignancy and suppressed the generation

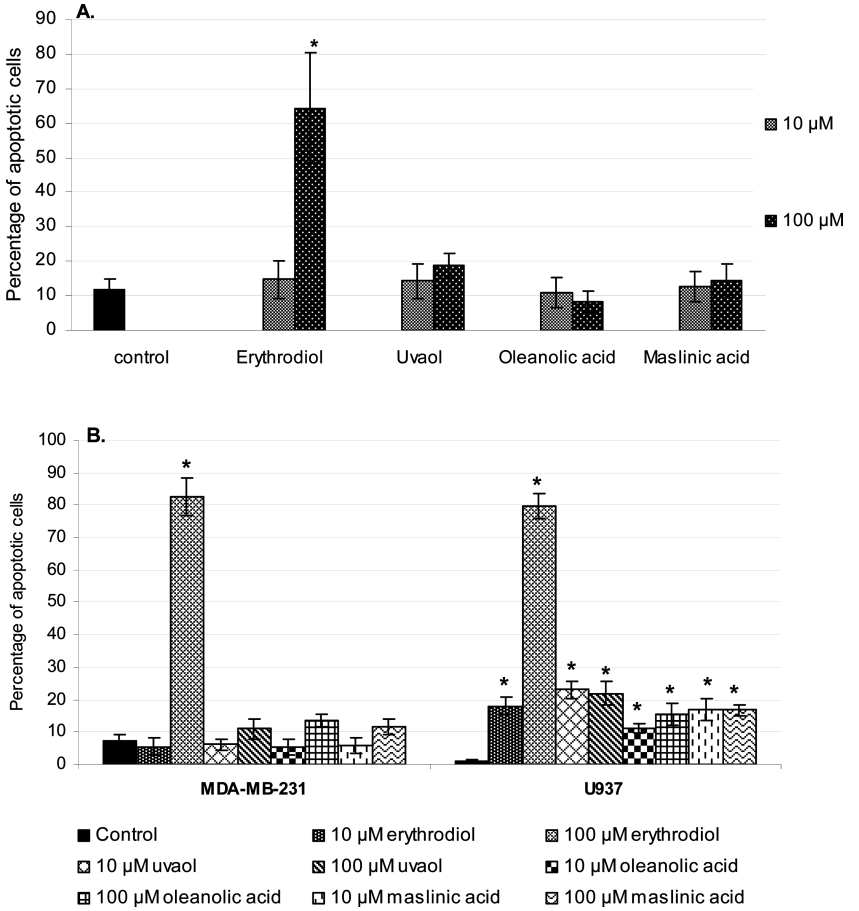

Figure 5. Effects of triterpenes on apoptosis induction on MCF-7 (**A**) and MDA-MB-231 and U937 cells (**B**). Cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were collected and stained with FITC-conjugated annexin V and PI. Following flow cytometry, the percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells using the FlowJo program. The results represent the mean $\pm$ SEM of three independent experiments. $*$ denotes statistically significant difference compared with control ($p < 0.05$).

potential of cancer cells. Uvaol showed a significant proliferative effect at the lowest dose of 12.5 μM. It has been reported that at low doses, certain antioxidants can stimulate the growth of some types of cancer. For instance, vitamin C was found to stimulate the growth of both human parotid carcinoma cells and human leukemic cells in vitro (*35*), and vitamin E was found to enhance the growth of various prostate cancer cell lines (*36*). Future work is needed to clarify the mechanism of stimulation of cancer cell growth by some antioxidants.

It has been reported that some anticancer agents cause growth inhibition through interfering with the processes of cell cycle (*37*) and some others cause cell death by apoptosis (*38*). On the one hand, cell cycle is under strict regulation in the cell with numerous control points that allow correct progression of the different phases. In this sense, a delay in progression of the G0/G1, S, or G2/M cell cycle phases would constitute a cellular defense mechanism to allow action of the DNA repair systems (*39*). On the other hand, apoptosis is considered to be a physiologically important process that functions to eliminate undesired cells during development and homeostasis of multicelular organisms. Therefore, to determine whether cell cycle arrest or apoptosis is involved in growth inhibition, we examined cell cycle phase distribution and induction of apoptosis of the triterpene-treated cells by flow cytometry. Our results indicated that at the concentration of 100 μM, uvaol and oleanolic acid inhibition of proliferation appeared to result from inhibition of cell cycle progression, because these compounds were found to be involved in the action of the G0/G1 checkpoint and inhibition of DNA replication. The literature lacks studies on the potential antiproliferative capacity of uvaol. Nonetheless, recently Martín et al. (*25*) reported that uvaol growth-inhibiting activity is associated with the induction of apoptosis in 1321N1 astrocytoma cells. These authors observed that exposure of 1321N1 cells to 50 and 100 μM uvaol for 18 h induces apoptosis around 40−60%. Instead, and in agreement with our results, Li et al. (*40*) and Cipak et al. (*41*) reported that oleanolic acid, at the concentration of 60 μM, inhibition of proliferation occurred through G0/G1 phase arrest, respectively, after 72 and 24 h of exposure in HCT-15 colon adenocarcinoma cell line and HL-60 leukemic cell line. By contrast, in another study it was found that oleanolic acid inhibition of proliferation is mediated via apoptosis in 1321N1 astrocytoma cells treated for 18 h with a 25 μM concentration of this triterpenic acid (*24*). Differences in uvaol and oleanolic acid responses are therefore most likely due to the specific cell type and concentration and treatment time used. Moreover, in our study, it was found that uvaol and oleanolic acid did not induce apoptosis in MDA-MB-231 human breast adenocarcinoma cell line but did in U937 human leukemic cell line, and it was not dose-dependent. Otherwise, our results suggest that 100 μM erythrodiol inhibition of MCF-7 cell proliferation appeared to result from the occurrence of apoptosis. Erythrodiol was also found to strongly induce apoptosis in MDA-MB-231 and U937 cells. Our findings are in agreement with others previously reported (*23*, *25*) describing that erythrodiol antiproliferative activity is associated with the induction of apoptosis and that erythrodiol apoptotic potential appears to be a generalized event.

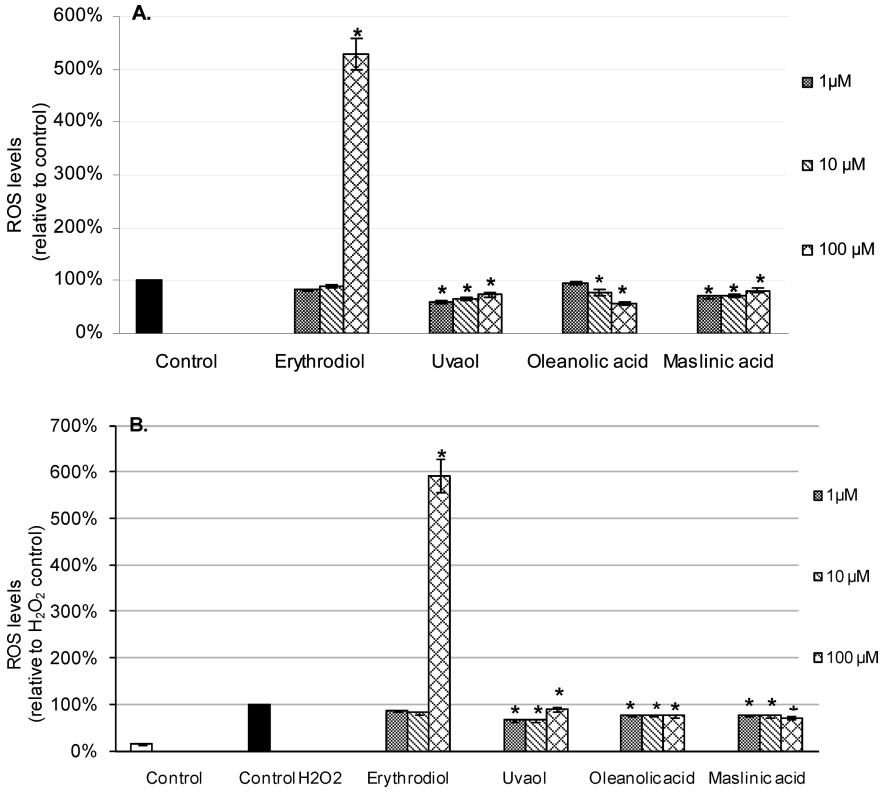

Figure 6. (**A**) Intracellular ROS level in MCF-7 cells after triterpene treatment. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then intracellular ROS level was measured by DCFH-DA probe. The results represent the mean ± SEM of three independent experiments. ∗ denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$). (**B**) Capacity of triterpenes to reduce MCF-7 oxidative stress induced by H_2O_2 addition measured by DCFH-DA probe. The results represent the mean ± SEM of three independent experiments. ∗ denotes statistically significant difference compared with positive control (control H_2O_2), which was considered to be 100% ($p < 0.05$).

Under our experimental conditions, incubation of MCF-7 cells for 24 h with maslinic acid did not exert any signs of cytotoxicity or alter cell cycle parameters, nor did it induce apoptosis. However, it increased proliferation rate. Our results are in contrast with others reported previously describing the antiproliferative and apoptosis-inducing effect of maslinic acid on colon and brain cancers (*21*, *22*, *24*, *42*, *43*). On the other hand, it was observed that maslinic acid induces apoptosis in U937 leukemic cells. Differences in susceptibility to maslinic acid may be therefore due to differences in the nature of the cell line. Further studies should be done to understand the mechanism by which maslinic acid stimulates MCF-7 cells proliferation.

It has been reported that many anticancer compounds exert antitumor activity including antioxidant mechanism (*44*). The triterpenes under study have been previously reported to possess antioxidant properties in a variety of experimental systems (*15*−*20*). However, to the authors' best knowledge, very little is known about the triterpenes' free radical scavenging activity. Using ABTS and DPPH chemical assays, our results indicated that the tested triterpenes lack free radical scavenging capacity. Accordingly, Yang et al. (*45*) reported that oleanolic and maslinic acids did not possess DPPH scavenging capacity up to the concentration of 200 μM. Nonetheless, in our study it was found that at very high mole ratio (up to 5.00 mol), maslinic acid exhibited a high DPPH scavenging capacity. Furthermore, we recently reported that maslinic acid, unlike the rest of the triterpenes, acts as an efficient peroxyl radical scavenger as assessed by the ORAC assay (*15*).

On the other hand, at present, overwhelming evidence indicates that ROS are involved in both the initiation and progression of cancer (*46*). In this sense, the cancer chemopreventive properties of antioxidants are generally believed to be due to their ability to scavenge endogenous ROS (*47*). Furthermore, oxidative stress may result in an increase in oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant system. In this paper, DCFH-DA assay results showed that uvaol and oleanolic and maslinic acids decreased significantly the steady-state generation of ROS by MCF-7 in culture. In addition, it was found that increased levels of ROS generated during the oxidative stress period (30 min of H_2O_2 exposure) were quenched in cells pretreated with these triterpenes. Overall, our data indicate that in the cellular system, uvaol and oleanolic and maslinic acids behaved as antioxidants, reducing both endogenous and exogenous ROS levels.

On the other hand, ROS levels have been shown to play an important role in the initiation and execution of apoptosis of many anticancer compounds (*48*). In this sense, erythrodiol, uvaol, oleanolic acid, and maslinic acid induction of apoptosis was found to be preceded by ROS generation in astrocytome cells (*24*, *25*). The same effect was observed by maslinic acid in HT-29 cells (*42*). On basis of these observations, in our study the increase in intracellular ROS level in MCF-7 cells treated with 100 μM erythrodiol may be related to apoptosis induction. Further experiments should be performed to study the detailed mechanism of apoptosis.

The comet assay provides a simple and effective method for evaluating DNA damage at the single-cell level. As expected, MCF-7 cell treatment with uvaol, oleanolic acid, or maslinic acid showed no genotoxicity at both doses tested. Similarly, 10 μM erythrodiol did not induce DNA damage. By contrast, and consistent with apoptosis and ROS results, 100 μM erythrodiol was found to produce high DNA damage.

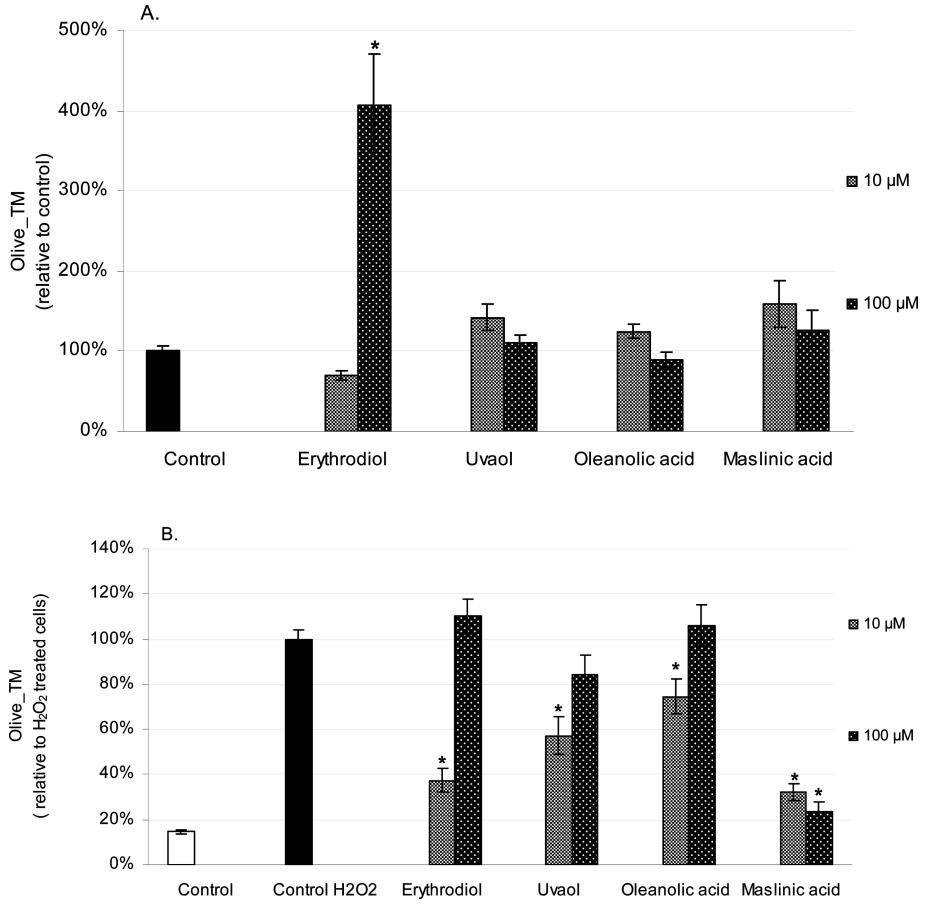

Figure 7. (**A**) Effects of triterpenes on DNA integrity assessed by the comet assay. MCF-7 cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were subjected to comet assay. Olive_TM values represent the mean ± SEM. * denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$). (**B**) Effects of triterpenes on DNA oxidative damage induced by H_2O_2 addition evaluated by the comet assay. Olive_TM values represent the mean ± SEM. * denotes statistically significant difference compared with positive control (control H_2O_2), which was considered to be 100% ($p < 0.05$).

H_2O_2 is suggested to cause DNA strand breakage by the generation of hydroxyl radicals ($OH^{\bullet}$) through the Fenton or Fenton-like reactions (*49*). In fact, in our study it was found that preincubation of MCF-7 cells with H_2O_2 for 10 min caused marked DNA damage. Very little is known regarding the effect of the tested triterpenes against DNA-induced oxidative injury, and only Ovesná et al. (*50*) have previously reported the effect of oleanolic acid against H_2O_2-induced DNA damage. These authors concluded that this triterpene significantly reduced DNA-induced oxidative injury in leukemic L1210, K562, and HL-60 cells in the concentration range from 2.5 to 10 μM. In agreement, in our experiment it was found that at 10 μM, oleanolic acid, as well as erythrodiol and uvaol, exhibited a protective effect against DNA damage caused by H_2O_2. However, no longer was a protective capacity observed when tested at 100 μM. By contrast, maslinic acid displayed a protective capacity at both doses tested.

According to Iliakis et al. (*51*), the DNA damage response is a hierarchical process; to allow time for DNA repair, the cells activate checkpoint pathways that delay the normal progression of the cell cycle. Checkpoints, together with repair and apoptosis, are integrated in a circuitry that determines the ultimate response of a cell to DNA damage. Therefore, our results, taken together, suggest that at low dose (10 μM), erythrodiol, uvaol, oleanolic acid, and maslinic acid in a dose-independent manner act as antioxidants scavenging superoxide and hydroxyls radicals produced by H_2O_2, thus preventing the impairment of mitochondrial function and consequently avoiding MCF-7 cell death. However, the absence of protective activity against DNA-induced damage in MCF-7 cells pretreated with high doses (100 μM) of erythrodiol, uvaol, and oleanolic acid may be linked to their capacity to activate other signaling pathways, resulting in cell cycle arrest and/or apoptosis and, thus, proliferation inhibition. Additional experiments to get a better understanding of the triterpenes' mechanisms of action are in progress.

In relation to the chemical structures, these pentacyclic triterpenes can be classified as alcoholic or acid (**Figure 1**). Although the structure and activity relationships of these pentacyclic triterpenes are far from clear, it seems that the $-CH_2OH$ group at C-28 enhances the cytotoxic capacity of the triterpenic diols as compared to triterpenic acids ($-COOH$ at C-28). In addition, the pro-apoptotic capacity of erythrodiol may be related to the presence of two methyl groups at C-20. Maslinic acid differs from the other triterpenes by the presence of an additional $-OH$ group at C-2, which appears to suppress the cytotoxic effect and confer it antioxidant activity.

To the authors' best knowledge, to date, there are no studies reporting the bioavailability of these triterpenes. Therefore, it is difficult to predict the presence of these compounds or their derived metabolites in the blood after consumption of a triterpene-rich diet. In any case, the concentrations used in the present study are similar to those previously reported (*21*−*25*).

In conclusion, our results provide new insight into the anticarcinogenic action of erythrodiol, uvaol, oleanolic acid, and maslinic acid in human breast cancer. Moreover, our findings

support the hypothesis that these triterpenes, isolated in appreciable higher amounts from the non-glyceride fraction of pomace olive oil, may be considered as valuable molecules for use as cancer chemotherapeutic or chemopreventive agents.

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Received for review June 16, 2010. Revised manuscript received November 16, 2010. Accepted November 23, 2010. This study was supported by the Ministerio de Ciencia e Innovación e INIA (RTA2008-00066-C03-03), Instituto Andaluz de Biotecnología (BIOÁNDALUS 08/22/L5.3), and Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud (CEAS).

Aceite de Oliva, Dieta Mediterránea y Salud

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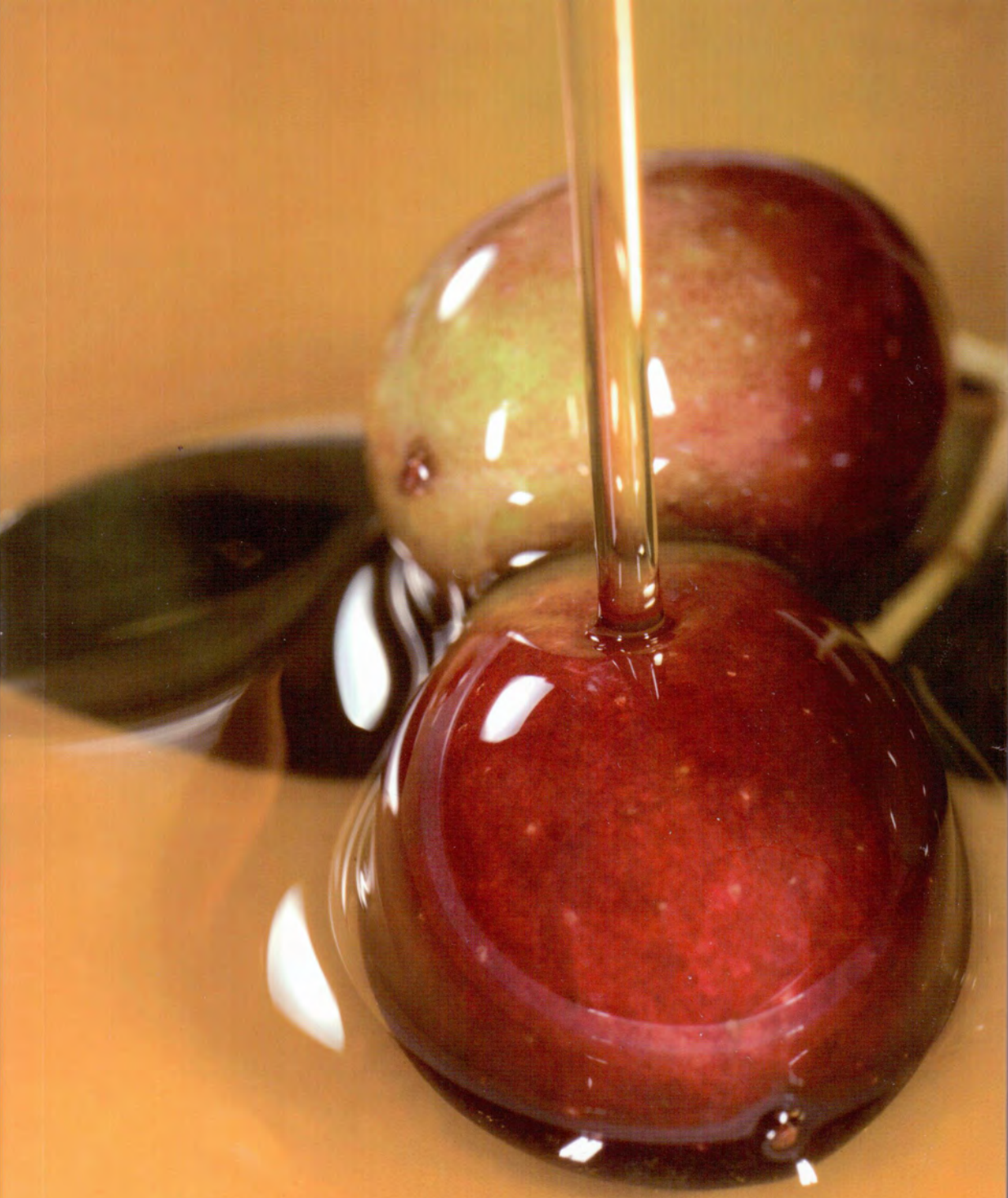
- Capítulo del libro: *"Ad oleum habendum"*
- Editorial: GEA Westfalia Separator Ibérica S.L.
- Editado por: Vilar Hernández, Juan
- Fecha de publicación: 2011
- Páginas: 295-322
- Idioma: Español
- ISBN: 978-84-615-0549-4



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Coordinadores: José Humanes, Juan Vilar, Manuel Fialho, Pilar Higueras

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Ministra de Medio Ambiente, Medio Rural y Marino

Coordinan:
José Humanes, Juan Vilar, Manuel Fialho, Pilar Higueras, Mª del Mar Velasco y Raquel Puentes.

Editan:
Cooperativa Agrícola de Moura e Barrancos,
CRL, Rua das Forças Armadas, 9, 7860-034 Moura (Portugal)
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Con la colaboración de la Cátedra Bancaja Jóvenes Emprendedores – Universidad de Jaén.

Depósito Legal: J-579-2011

I.S.B.N.: 978-84-6150549-4

Diseña e imprime:
Gráficas Francisco del Moral, s.a.
www.graficasdelmoral.com

CAPÍTULO 13
ACEITE DE OLIVA, DIETA MEDITERRÁNEA Y SALUD

José Juan Gaforio, Fernando Warleta y Cristina Sánchez

1. CARACTERÍSTICAS DE LAS GRASAS. GRASAS VEGETALES Y ANIMALES

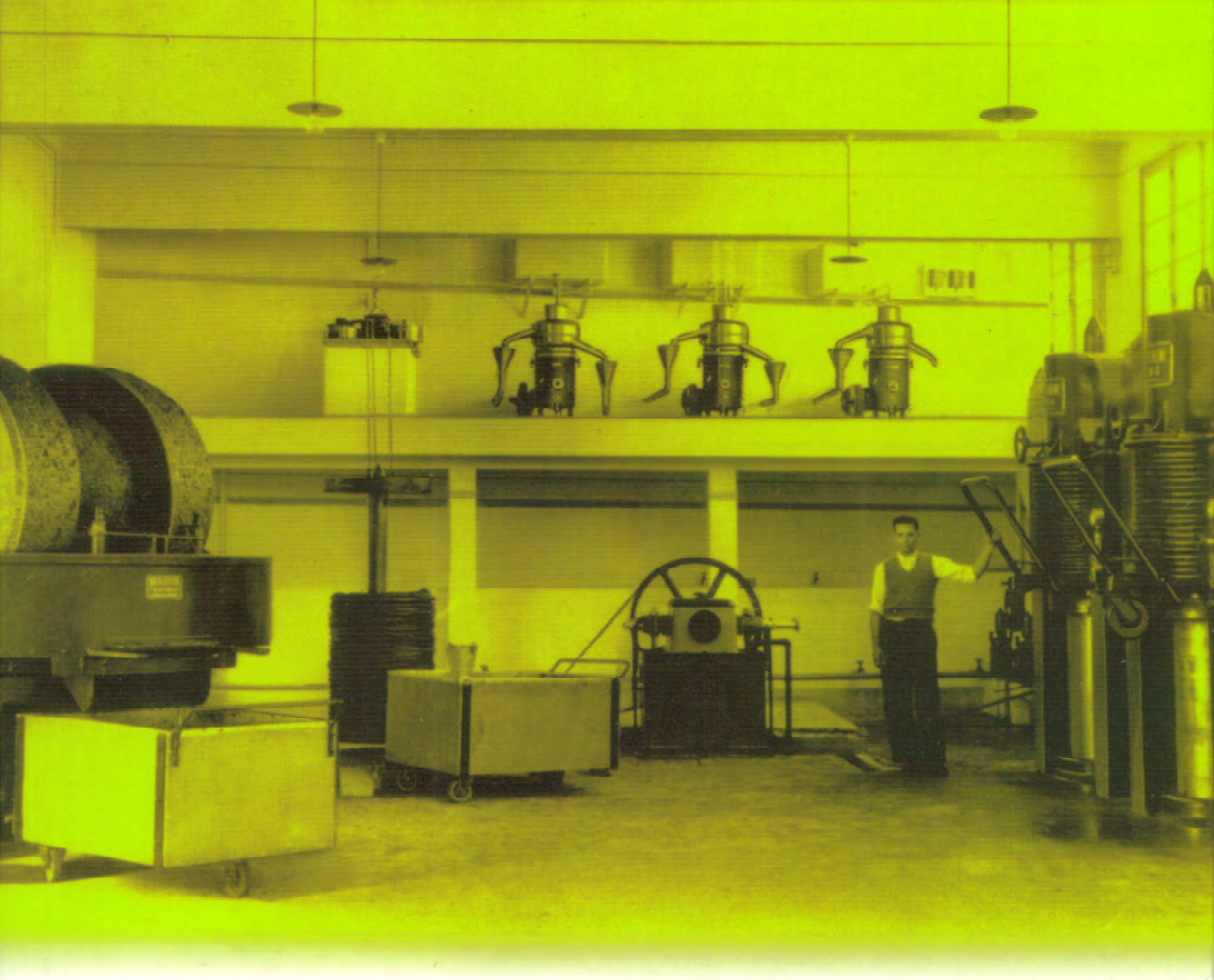
Las grasas o lípidos ingeridos en nuestra dieta, son vitales para mantener el estado de salud, por tanto, es sano consumir grasas. En este sentido, hay que tener en consideración los siguientes datos:

- Las grasas son la principal fuente de energía en la dieta, aportan 9 Kcal por cada gramo consumido, frente a las 4 Kcal que proporcionan los hidratos de carbono y las proteínas.
- Representan un factor clave en el mantenimiento del equilibrio calórico y del peso de nuestro organismo. Los depósitos de grasa en el cuerpo humano representan una reserva de energía para ser utilizada en caso necesario; protegen y rodean los órganos vitales y contribuyen a aislar nuestro organismo del frío.
- Facilitan la absorción de las vitaminas liposolubles: A, D, E y K así como de los carotenoides.
- Los ácidos grasos esenciales (ácido linoleico y ácido álfa-linolénico) no pueden ser sintetizados por nuestro organismo y deben ser aportados en la dieta. Son necesarios para el crecimiento y desarrollo normalizado de nuestro organismo.
- Ciertos ácidos grasos intervienen como precursores en numerosas vías biológicas implicadas en los procesos de inflamación, coagulación y expresión génica, entre otras funciones.
- Son fundamentales en la formación y funcionalidad de las membranas celulares.
- A todo ello hay que sumar que, las grasas aportan a los alimentos

Olive Oil, Mediterranean Diet and Health

José Juan Gaforio, Fernando Warleta y Cristina Sánchez

- Capítulo del libro: *"Culture and Knowledge Inspired by Olive Oil"*
- Editorial: GEA Westfalia Separator Ibérica S.L.
- Editado por: Vilar Hernández, Juan
- Fecha de publicación: 2011
- Páginas: 331-356
- Idioma: Inglés
- ISBN: 978-84-615-0550-0



CULTURE and knowledge

INSPIRED BY OLIVE OIL

Foreword by Jean-Louis Barjol
Coordinators: José Humanes, Juan Vilar, Manuel Fialho and Pilar Higueras

CULTURE AND KNOWLEDGE INSPIRED BY OLIVE OIL

Cover photograph:

Mixed olive oil mill operated by pressure (Italian style-millstones) and separation (vertical separators Westfalia Separator), olive oil mill in Italy in the fiftees. Source: GEA Westfalia Separator Group.

Back Cover photograph:

Juan Vilar Rodríguez, Juan Vilar Velasco y Juan Vilar Hernández (three generations of olive farmers) in olive field "Las Monjas", Chilluévar (Jaén – Spain).

FOREWORD:

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www.azeitemoura.pt

With the collaboration of Cátedra Bancaja Jóvenes Emprendedores – University of Jaén

I.S.B.N.: 978-84-615-0550-0

Legal Deposit:

J-581-2011

Design and print:

Gráficas Francisco del Moral, s.a.
www.graficasdelmoral.com

CHAPTER 14

OLIVE OIL, MEDITERRANEAN DIET AND HEALTH

José J. Gaforio,
Fernando Warleta
and Cristina Sánchez

1. CHARACTERISTICS OF FATS. ANIMAL AND VEGETABLE FATS

The fats or lipids ingested in our diet are vital to maintain our health condition; therefore it is healthy to consume fats. In this sense, we have to take into consideration the following data:

- Fats are the main source of energy in our diet, they provide 9 Kcal per gram consumed, compared to the 4 Kcal provided by carbohydrates and proteins.
- They represent a key factor in the maintenance of the caloric balance and weight of our organism. The fat deposits in the human body represent an energy reserve that can be used whenever necessary; they protect and surround vital organs and contribute towards isolating our organism from the cold.
- They facilitate the absorption of liposoluble vitamins: A, D, E and K as well as carotenoids.
- Essential fatty acids (linoleic acid and alpha-linolenic acid) can not be synthesised by our organism and must by provided in the diet. They are necessary for the growth and normal development of our organism.

Phenotypic and genetic characterization of circulating tumor cells by combining immunomagnetic selection and FICTION techniques

Campos M., Prior C., Warleta F., Zudaire I., Ruíz-Mora J., Catena R., Calvo A., Gaforio, J.J.

- Trabajo enviado a la revista *"Journal of Histochemistry & Cytochemistry"*
- Fecha de publicación: Julio de 2008
- Nº de citas (a 1 de Diciembre de 2015): 10
- SCImago Journal & Country Rank
  - Categoría perteneciente: Histology
  - Índice SJR: 1,062
  - H index: 97
  - Posición: 10/57
  - Cuartil en la categoría: Q1
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Cell Biology
  - Índice de impacto: 1,959
  - Posición: 147/184
  - Cuartil en la categoría: Q4

Volume 56(7): 667–675, 2008
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Phenotypic and Genetic Characterization of Circulating Tumor Cells by Combining Immunomagnetic Selection and FICTION Techniques

María Campos, Celia Prior, Fernando Warleta, Isabel Zudaire, Jesús Ruíz-Mora, Raúl Catena, Alfonso Calvo, and José J. Gaforio

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SUMMARY The presence of circulating tumor cells (CTCs) in breast cancer patients has been proven to have clinical relevance. Cytogenetic characterization of these cells could have crucial relevance for targeted cancer therapies. We developed a method that combines an immunomagnetic selection of CTCs from peripheral blood with the fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm (FICTION) technique. Briefly, peripheral blood (10 ml) from healthy donors was spiked with a predetermined number of human breast cancer cells. Nucleated cells were separated by double density gradient centrifugation of blood samples. Tumor cells (TCs) were immunomagnetically isolated with an anti-cytokeratin antibody and placed onto slides for FICTION analysis. For immunophenotyping and genetic characterization of TCs, a mixture of primary monoclonal anti-pancytokeratin antibodies was used, followed by fluorescent secondary antibodies, and finally hybridized with a TOP2A/HER-2/CEP17 multicolor probe. Our results show that TCs can be efficiently isolated from peripheral blood and characterized by FICTION. Because genetic amplification of *TOP2A* and *ErbB2* (*HER-2*) in breast cancer correlates with response to anthracyclines and herceptin therapies, respectively, this novel methodology could be useful for a better classification of patients according to the genetic alterations of CTCs and for the application of targeted therapies. **(J Histochem Cytochem 56:667–675, 2008)**

KEY WORDS
breast cancer
circulating tumor cells
cytokeratin expression
FICTION
ERBB2 (*HER-2/neu*) gene
immunomagnetic selection
TOP2A gene

BREAST CANCER is the most frequent type of cancer in women (Parkin et al. 2005). Because of its high incidence and good prognosis, breast cancer is the most prevalent cancer in the world today. Approximately 4.4 million women who were diagnosed with breast cancer within the last 5 years are still alive (Parkin et al. 2005). Despite the improvement in detection and treatment, ~30% of newly diagnosed women with breast cancer will die. In most cases, death results from the dissemination of cancer cells through lymphatic or blood vessels and the development of distant metastases.

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Received for publication February 22, 2008; accepted March 27, 2008 [DOI: 10.1369/jhc.2008.951111].

The *ErbB2* oncogene (also known as *c-erbB2/HER-2/neu*) is the most frequently amplified oncogene in breast cancer (20–35% of invasive breast tumors) (Pauletti et al. 1996; Press et al. 1997; Ross and Fletcher 1999), which correlates with poor clinical outcome (Järvinen and Liu 2003). *ErbB2* is localized at 17q12-q21 and encodes a 185-kDa tyrosine kinase protein that belongs to the epidermal growth factor (EGF) receptor family. Although there is no known ligand for *ErbB2*, heterodimerization with other members of the EGF receptor family causes a strong mitotic response (Olayioye et al. 2000; Yarden and Sliwkowski 2001). Patients with *ErbB2* gene amplification or protein overexpression are eligible for trastuzumab (Herceptin) therapy.

TOP2A, a gene that codes for the protein topoisomerase-IIα, is another key gene for breast cancer. *TOP2A* is located at the *ErbB2* region and has been found altered in almost 90% of primary breast

0022-1554/08/$3.30

cancers with *ErbB2* amplification (Järvinen et al. 1999, 2000). However, although 40% of those cases have a coamplification of both genes, in another 40% of the tumors, *TOP2A* has been found deleted (Järvinen and Liu 2003). Breast cancer cells with high topoisomerase-IIα expression show a better response to anthracyclines (such as doxorubicin, epirubicin, or idarubicin) (Järvinen and Liu 2003,2006; Hannemann et al. 2006). Therefore, the determination of genomic amplification of either *ErbB2* or *TOP2A* in breast cancer patients is critical for administering an effective targeted therapy.

Breast cancer has been shown to shed tumor cells into the circulation, even at the earliest stages of primary tumor development (Gaforio et al. 2003). Pretlow et al. (2000) have shown that circulating tumor cells (CTCs) isolated from peripheral blood (PB) of patients with cancer are able to develop metastasis when xenotransplanted into nude mice. Thus, the early detection of CTCs may have important therapeutic and prognostic implications. The number of CTCs in PB from breast cancer patients has clinical relevance (Gaforio et al. 2003; Cristofanilli et al. 2005; Cristofanilli and Mendelsohn 2006; Müller et al. 2006; Camara et al. 2007), not just as an indicator of overall survival, but also as a disease progression marker and/or metastatic marker. However, little attention has been paid to the cytogenetic features of such cells.

In 1992, Weber-Matthiesen et al. (1992) developed a new technique for simultaneous immunophenotyping and interphase cytogenetics analyses using cell lines. The method was referred to as fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm (FICTION). This technique was shown to be very useful in the study of hematological neoplasms (Nylund et al. 1993; Haferlach et al. 1997; Temple et al. 2004; Young et al. 2006). In this study, we show the development of a novel method that combines immunomagnetic selection of tumor cells (TCs) with cytogenetic characterization by FICTION analysis. With this method, we were able to isolate TCs from a 10-ml blood sample, immunophenotype them (with anti-cytokeratin antibodies), and analyze *ErbB2*, *TOP2A*, and *CEP17* gene copy number.

Materials and Methods

Materials

Cell culture media MEM with Earle's salt and RPMI-1640 and fetal calf serum (FCS) were obtained from PAA Laboratories (Pasching, Austria). Penicillin-streptomycin, PBS, formaldehyde 37% solution (formalin), Igepal, Histopaque 1077, Histopaque 1119, poly-L-lysine–coated glass slides, and Fast Red TR/Naphthol AS-MX substrate were purchased from Sigma (St. Louis, MO). The Carcinoma Cell Enrichment and Detection Kit, the MACS MS Columns, anti-cytokeratin 7/8 antibody (isotype: mouse IgG2a) conjugated to FITC, and anti-FITC antibody (Isotype: mouse IgG1) conjugated to alkaline phosphatase were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Mouse anti-human AE1-AE3 antibody was purchased from BioGenex (San Ramon, CA). This is a biclonal mouse antibody cocktail that recognizes cytokeratins 1/2/3/4/5/6/7/8 (clone: AE1; immunoglobulin class: IgG1) and cytokeratins 10/14/15/16/19 (clone: AE3; immunoglobulin class: IgG1). Secondary antibodies Alexa Fluor 350 rabbit anti-mouse IgG (H+L) and Alexa Fluor 350 goat anti-rabbit IgG (H+L) and the Slow Fade Light Antifade Kit were obtained from Invitrogen (Eugene, OR). The LSI TOP2A/HER-2/CEP17 multicolor probe was purchased from Abbott Molecular (Vysis; Des Plaines, IL). Sodium chloride-sodium citrate buffer (SSC) was obtained from MP Biomedicals Europe (Illkirch, France). Methanol, acetone, and ethanol absolute were purchased from Panreac Quimica (Barcelona, Spain). Acetic acid (glacial), Mayer's hematoxylin, and Kaiser's glycerol gelatin were obtained from Merck (Darmstadt, Germany).

Cell Lines

The human breast cancer cell lines MCF-7 and MDA-MB-231, known to have no *ErbB2* amplification, were obtained from the American Type Cultured Collection (ATCC; Rockville, MD). SK-Br-3, a human breast cancer cell line with *ErbB2* amplification, was obtained from Eucellbank (Barcelona, Spain). MCF-7 and MDA-MB-231 cells were grown in MEM with Earle's salt, supplemented with 10% (v/v) heat-inactivated FCS and 1% of a stock solution of penicillin-streptomycin. SK-Br-3 was grown in RPMI 1640, supplemented with 10% (v/v) heat-inactivated FCS and 1% of a stock solution of penicillin-streptomycin. Cells in the exponential growth phase were used for all experiments.

Blood Sample Processing and Density Gradient Separation

PB samples (10 ml) from nine healthy volunteers were obtained in heparinized tubes (BD Vacutainer; Becton Dickinson, Heidelberg, Germany) and spiked with 1000 MCF-7, MDA-MB-231, or SK-Br-3 breast cancer cells. As negative controls, we processed three different samples without breast cancer cells. All samples were obtained with previous informed consent of healthy donors. A double-density ficoll gradient was prepared by placing 5 ml Histopaque 1077 in a tube containing an equal volume of Histopaque 1119. Blood samples were carefully placed onto the upper layer of Histopaque 1077, and tubes were centrifuged at 700 × g for 30 min at 20C. The mononuclear cell fraction (plasma/1077 interphase) and granulocyte fraction (1077/1119 interphase) were isolated, and both fractions were

mixed and washed in 50 ml PBS by centrifugation at 200 × g for 15 min, according to previous publications (Gaforio et al. 2003).

Cell Enrichment by Immunomagnetic Separation

Tumor cells were immunoseparated using the Carcinoma Cell Enrichment and Detection Kit, with some modifications. Briefly, after cell permeabilization, the cell pellet was resuspended in 40 ml dilution buffer plus 5 ml MACS CellPerm Solution and incubated for 5 min at room temperature. Cells were fixed by adding 5 ml CellFix Solution for 30 min at room temperature. Cells were washed twice, resuspended in 600 µl MACS CellStain Solution, incubated with 200 µl Fc-Blocking Reagent, and immunomagnetically labeled with 200 µl MACS anti-cytokeratin microbeads (microbeads conjugated to a monoclonal anticytokeratin 7/8 antibody; clone: CAM5.2).

After a 45-min incubation at room temperature, cells were washed, resuspended in 1 ml CellStain Solution, and placed onto a MACS MS column. Unlabeled cells were washed off the column with 3 × 500 µl dilution buffer. The column was removed from the magnetic field, and the retained cells (magnetic-positive cell population) were eluted with 1 ml dilution buffer. The magnetically enriched cell fractions were spun down onto poly-L-lysine–coated glass slides in a cytocentrifuge (Hettich; Tuttlingen, Germany) at 1500 rpm for 10 min. Slides were air-dried overnight at room temperature and stored at −20C without fixation.

LSI TOP2A/HER-2/CEP17 Probes

The LSI TOP2A/HER-2/CEP17 multicolor probe includes a TOP2A probe labeled with SpectrumOrange, an ERBB2 (HER-2/neu) probe labeled with SpectrumGreen, and a chromosome enumeration probes CEP17, labeled with SpectrumAqua.

FICTION

Previously published protocols for FICTION were used (Weber-Matthiesen et al. 1992), with some modifications. After thawing, slides were fixed in an ice-cold mixture of methanol and acetone (1:1) for 5 min and air dried. Slides were hydrated in PBS for 5 min and incubated for 30 min with a blocking solution (10% rabbit serum in PBS) before primary antibody incubation. The slides were washed in PBS for 5 min and incubated with the biclonal mouse anti-AE1-AE3 antibody overnight at 4C, diluted 1:200 in PBS containing 10% FCS. The revelation was conducted by applying two sequential layers of secondary antibodies (1:50 dilution in PBS for both of them, 30 min at room temperature): Alexa Fluor 350 rabbit anti-mouse IgG and goat anti-rabbit IgG. After fluorescent immunophenotyping, slides were coverslipped under PBS for evaluation under a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss; Jena, Germany), with appropriate filter sets. ISIS software (MetaSystems; Altslussheim, Germany) was used for evaluation throughout.

After assessment of positive (tumor) cells on the slides, samples were fixed with Carnoy's solution (3:1 methanol: acetic acid fixative), rinsed in distilled water for 1 min, fixed in 1% formaldehyde, and rinsed again in distilled water for 1 min. After dehydration in an ethanol series (70%, 80%, and 100%) and air dried, slides were codenatured with the LSI TOP2A/HER-2/CEP17 multicolor probe for 5 min at 85C and hybridized overnight in a humidified chamber at 37C. Posthybridization wash was carried out at 72C in 2× SSC/0.3% Igepal, pH 7, for 2 min, followed by another wash at room temperature. Finally, slides were mounted with the Slow Fade Light Antifade Kit. Microscopic evaluation was carried out with the microscope and software imaging system described above.

A minimum number of 50 morphologically intact and non-overlapping nuclei were scored in every sample to determine the number of hybridization signals for each ErbB2, TOP2A, and CEP17 probe. Both absolute copy numbers and the relative copy number ratio (ratio between the mean number of ErbB2 or TOP2A signals and the mean number of chromosome 17 centromere signals) were determined. A [ErbB2 or TOP2A]/CEP17 ratio ≥1.5 was considered gene amplification. Similarly, ratios ≤0.7 were considered gene deletions.

Analysis of Cell Recovery

To determine the number of cells recovered, we applied the double-density ficoll gradient and immunomagnetic separation describe previously, followed by immunocytochemistry (using anti-cytokeratin antibodies) for quantification of positive cells. Briefly, we used 10 ml of blood from healthy volunteers that was spiked with 1000 cells (MCF-7, MDA-MB-231, or SK-Br-3 cell lines). After double-density ficoll gradient, immunomagnetic labeling, and separation, cells were subjected to cytospin and stored at −20C without fixation. After thawing, slides were stained with 100 µl of anti-cytokeratin 7/8 conjugated to FITC (isotype: mouse IgG2a) for 10 min at room temperature and further labeled with 10 µl anti-FITC antibody conjugated to alkaline phosphatase (isotype: mouse IgG1) for 10 min at room temperature. Slides were washed in PBS for 5 min, and cytokeratin-expressing cells were detected by incubation with Fast Red TR/Naphthol AS-MX substrate solution for 15 min in a moist chamber. Slides were washed in PBS, counterstained with Mayer's hematoxylin, and mounted with Kaiser's glycerol gelatin. Cytokeratin-expressing cells (CK+) were counted separately by two expert researchers. All experiments were done in triplicate.

Results

Efficiency of the TCs Immunoselection Method

Cell recovery was in the range of 60–80% for the three cell lines studied (Table 1), which is similar to what was described in previous studies (Gaforio et al. 2003). All tumor cells (CK+) showed a strong cytoplasmic staining pattern, and the cell morphology was consistent with a malignant phenotype (Figures 1A–1C). According to previous publications, although tumor cells were specifically immunoselected, some white blood cells could be seen in the positive tumor fraction (Figures 1A–1C). Immunophenotyping was also conducted with mouse anti-cytokeratins antisera followed by two layers of fluorescent antibodies (Alexa Fluor 350 rabbit anti-mouse and Alexa Fluor 350 goat anti-rabbit). This procedure rendered an excellent labeling of tumor cells for the three lines analyzed (Figures 1D–1F).

FICTION Analysis of the Selected TCs

Breast cancer cells were unequivocally distinguished among the white blood cells by their blue color provided by the immunofluorescent labeling (Figures 2A–2C). The three tumor cell lines displayed a well-preserved morphology (Figures 2A–2C).

Hybridization signals for ErbB2, TOP2A, and CEP17 were also clearly observed in both tumor cells and leukocytes (Figures 2A–2F). All leukocytes showed two signals for ErbB2, TOP2A, and CEP17, thus serving as internal controls. Quantitative results of the number of signals found in tumor cells are given in Table 2. The most common genetic pattern for the cell line MCF-7 was the presence of three copies of chromosome 17 (CEP17) but two copies of *ErbB2* (mean, 2.02 ± 0.24 copies/cell) and *TOP2A* (mean, 2.01 ± 0.26 copies/cell; Figure 2D; Table 2). In the case of MDA-MB-231 cells, two patterns were basically found. The predominant one (accounting for 91.33% of the cells) showed trisomy of CEP17. The other one was tetrasomic for CEP17 (which was observed in 8.67% of the cells). In general, neither of these populations presented *ErbB2* or *TOP2A* amplifications or deletions (average ratio with respect to CEP17 being 1.01 for both genes; Table 1). The cell line SK-Br-3 showed many copies of *ErbB2*, *TOP2A*, and CEP17 (Figure 2F). The average copy number for *ErbB2* was 25.87 ± 7.39, whereas for *TOP2A*, it was 9.48 ± 2.55. Gene amplification for *ErbB2* (3.81-fold relative to CEP17) was found in this cell line. In negative control samples, no hybridization signals or blue-positive cells were detected.

Discussion

Studies from our group and from others have shown that the number of CTCs in breast cancer patients correlates with clinical outcome (Gaforio et al. 2003; Cristofanilli et al. 2005; Müller et al. 2006). The presence of more than five CTCs in a 7.5-ml blood volume determines a bad prognosis and decreased overall survival, irrespective of the treatment received by the patients (Cristofanilli et al. 2004). These remarkable data could be even more clinically relevant if the precise genetic alterations of such cells were accurately determined. We showed here the feasibility of analyzing TCs by immunomagnetic separation and FICTION techniques to examine gene copy number of *ErbB2*, *TOP2A*, and CEP17 in CK+ (i.e., tumor) cells.

Few studies have applied molecular techniques to identify genetic signatures of CTCs. The analysis by RT-PCR for mammaglobin and B305D-C in CTCs showed a sensitivity of 70% and a specificity of 81% for the diagnosis of invasive breast carcinoma (Reinholz et al. 2005). Several studies analyzing genetic defects in disseminated tumor cells isolated from bone marrow showed several chromosomal alterations (Klein et al. 1999; Schmidt-Kittler et al. 2003) and amplification of the *ErbB2* gene by fluorescence in situ hybridization (FISH) (Müller et al. 1996; Schardt et al. 2005). However, the molecular characterization of CTCs in PB would be preferable to more invasive procedures, such as bone marrow aspirations.

The FICTION technique was developed to assign tumor cells to a cytogenetically defined clone and, at the same time, to determine their specific cell lineage (Weber-Matthiesen et al.1992,1993). FICTION has been proven to be clinically meaningful in hematological neoplasms (Nylund et al 1993; Haferlach et al. 1997; Temple et al. 2004; Young et al. 2006). In solid tumors, three studies have applied FICTION to correlate the presence of genetic alterations with the ex-

Table 1 Recovery rates of tumor cells from peripheral blood

	Recovery rates[a]			
Cell line	Number of tumor cells recovered in blood sample 1	Number of tumor cells recovered in blood sample 2	Number of tumor cells recovered in blood sample 3	Total percentage of recovery
SK-Br-3	780	760	751	76.37
MCF-7	681	733	742	71.87
MDA-MB-231	670	657	622	64.97

[a]To determine the number of cells recovered, 10 ml of blood from nine healthy donors was spiked with 1000 cells (MCF-7, MDA-MB-231, or SK-Br-3 cell lines). See Analysis of Cell Recovery in Materials and Methods.

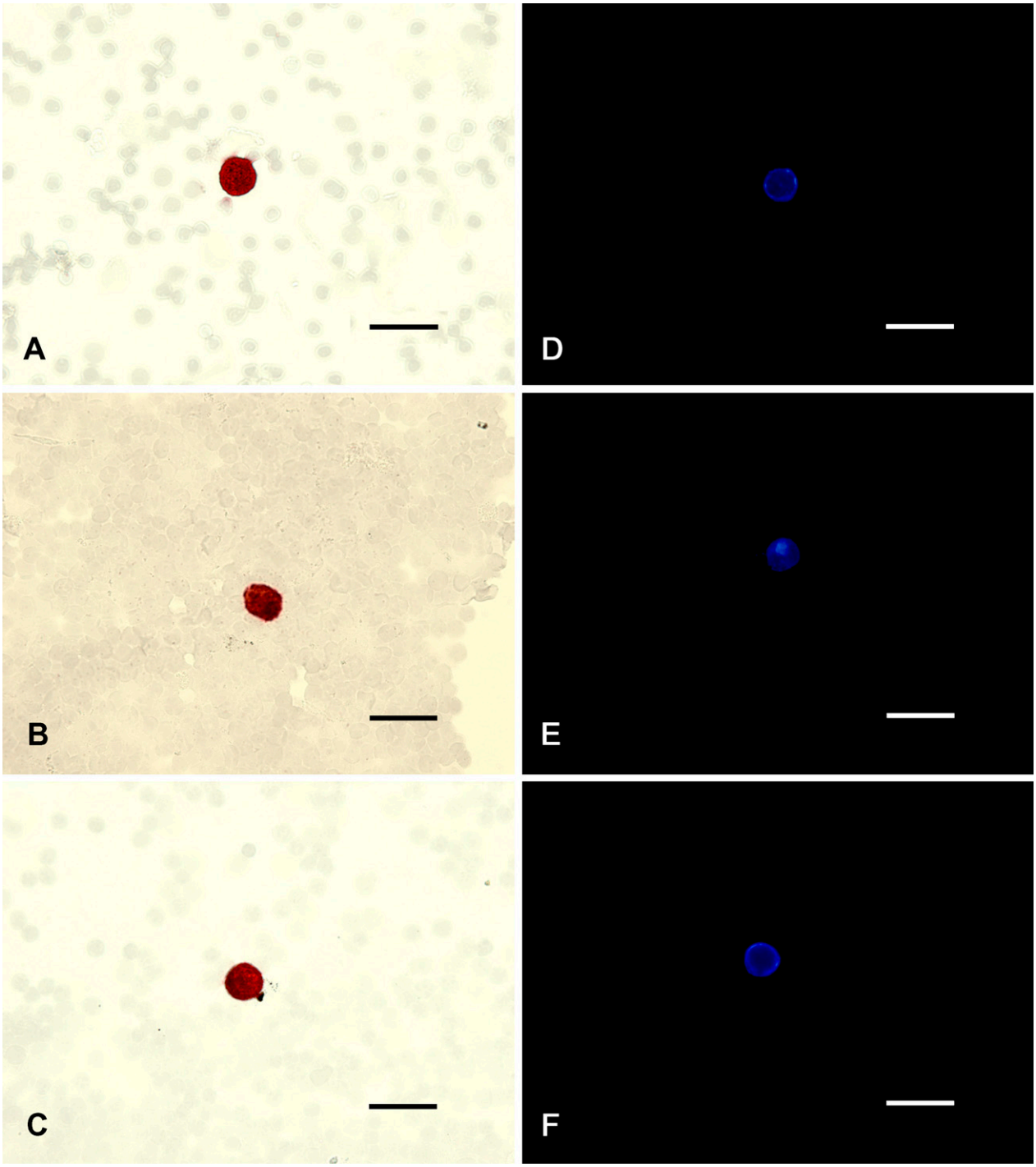

Figure 1 Peripheral blood from healthy volunteers spiked with different tumor cell lines. Mononuclear and granulocyte cell fractions were processed for positive immunomagnetic tumor cells separation and labeled using immunocytochemistry (**A–C**) or immunofluorescence (**D–F**). Tumor cells [MCF-7 (**A,D**), MDA-MB-231 (**B,E**), and SK-Br-3 (**C,F**)] show a solid cytoplasmic cytokeratin (CK) staining pattern, whereas the surrounding hematopoietic cells do not. Bar = 75 μm.

pression of a particular protein (Terada et al. 2000; Zhang et al. 2000; Dettori et al. 2003). Zhang et al. (2000) used this technique in six breast carcinoma cell lines to study the relationship between estrogen receptor (ER) expression and deletion of the estrogen receptor gene (*ESR*). Terada et al. (2000) studied, using FICTION, 105 patients with gastric tumors, showing that the frequency of positive cells for proliferating cell

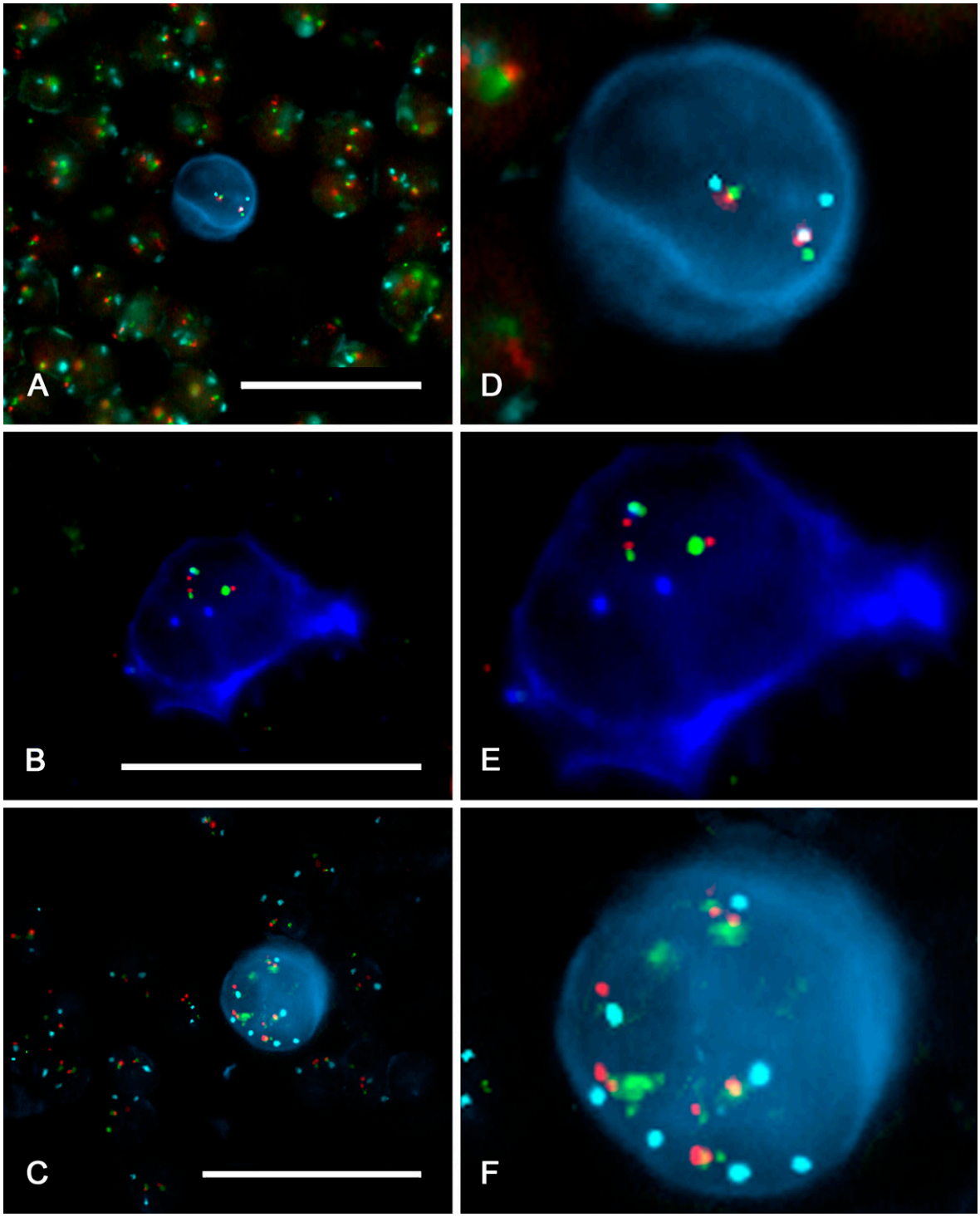

Figure 2 Peripheral blood from healthy volunteers spiked with different tumor cell lines, processed by immunomagnetic enrichment and fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm (FICTION) techniques. Leukocytes are negative for the CK labeling and were used as positive control for the fluorescence in situ hybridization technique. Tumor cells [MCF-7 (**A,D**), MDA-MB-231 (**B,E**), and SK-Br-3 (**C,F**)] are clearly identified and show hybridization signals for ERBB2 (HER-2/neu) (green), TOP2A (orange), and CEP17 [aqua (light blue)]. (**D**) MCF-7 cells show three copies of chromosome 17 and two copies of the *HER-2* and *TOP2A* (mean: two copies of both genes/cell). (**E**) The general pattern found for MDA-MB-231 cells is the presence of three signals for CEP17, HER-2, and TOP2A. (**F**) SK-Br-3 cells show a high level of *HER-2* amplification and a low level of *TOP2A* amplification. Observe that several signals are also seen for CEP17. Notice that not all signals from genes are in focus at the same time. Bar = 75 μm.

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Table 2 Absolute and relative copy numbers of *ERBB2* (*HER-2/neu*) and *TOP2A* genes in SK-Br-3, MCF-7, and MDA-MB-231 cells, after immunomagnetic separation from blood samples and FICTION analyses

	ERBB2 (*HER-2/neu*) copy number		*TOP2A* copy number	
Cell line	Absolute (mean ± SD)	Relative to 17 centromere	Absolute (mean ± SD)	Relative to 17 centromere
Leukocytes[a]	2.00 ± 0.00	1.00	2.00 ± 0.00	1.00
SK-Br-3	25.87 ± 7.39	3.81[b]	9.48 ± 2.55	1.40[b]
MCF-7	2.02 ± 0.24	0.66[c]	2.01 ± 0.26	0.66[c]
MDA-MB-231	3.09 ± 0.28	1.01	3.09 ± 0.28	1.01

[a]Samples internal control, 450 cells.
[b]Gene amplification.
[c]Gene deletion.
One hundred fifty cells were evaluated for each cell type (50 cells/sample). FICTION, fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm.

nuclear antigen (PCNA) and chromosome 17 numerical aberrations were indicators of metastatic potential. Dettori et al. (2003) evaluated, using FICTION, the immunoexpression of a mitochondrial membrane antigen with a simultaneous visualization of numerical chromosomal aberrations in 18 patients with thyroid tumors.

In this study, we showed for the first time that breast cancer cells isolated from PB can be analyzed by FICTION to simultaneously examine ErbB2/TOP2A/CEP17 copy number and cytokeratins 1-8/10/14-16/19 in these cells. To ensure a high specificity of the assay in the analysis of tumor cells, we incorporated the following steps: (a) the first milliliters of blood collected from the samples were discarded to avoid contamination with squamous cells; (b) Fc receptors from cells were blocked to avoid nonspecific binding, previously to the incubation with the anti-cytokeratin mAb; and (c) application of double CK immunolabeling with an anti-CAM5.2 (cytokeratin 7/8) monoclonal antibody (for immunoselection) and with a biclonal anti-AE1/AE3 (anti-pancytokeratin) antibody (for further immunophenotyping).

The genetic amplification of both *ErbB2* and *TOP2A* in SK-Br-3 cells was previously published using FISH (Szöllösi et al. 1995; Järvinen et al. 1999,2000; Forozan et al. 2000), comparative genomic hybridization (Forozan et al. 2000; Lottner et al. 2005), and spectral karyotyping (Kytölä et al. 2000), and is coincident with our results. The deletion of *ErbB2* in MCF-7 cells (Szöllösi et al. 1995; Shadeo and Lam 2006) and the normal *ErbB2* copy number in MDA-MB-231 cells (Satya-Prakash et al. 1981; Grushko et al. 2002; Lottner et al. 2005) were also previously documented and are in keeping with our data. However, no data showing the *TOP2A* status in MCF-7 and MDA-MB-231 cells were previously reported.

Ideally, the technique we describe here could be useful for early detection of *ErbB2/TOP2A*-amplified disseminated breast cancer and to monitor, in a relatively easy way, the response to herceptin/anthracycline-based therapies. However, many questions arise from our study that should be addressed in future studies. First of all, breast cancer patients should be tested to determine the clinical value of this technique. In addition, it is currently unknown whether *ErbB2/TOP2A* amplifications or deletions in CTCs will represent the general pattern of aberrations of the primary tumor. *ErbB2* is amplified in ~20–35% of invasive breast carcinomas, and *TOP2A* has been found altered in almost 90% of *ErbB2*-amplified tumors. However, several tumors with extra copies of *ErbB2* contain tumor cells with amplification and deletion of the *TOP2A* gene (Järvinen et al. 1999). It has been suggested that this finding may have therapeutical implications, because *TOP2A* deletions confer resistance to topoisomerase II inhibitors (Järvinen and Liu 2003). This could be an explanation for the gradual loss of efficacy of topoisomerase II inhibitors, which is common in breast cancer treatment. Meng et al. (2004) found that 9 of 24 patients whose primary tumors were *ErbB2* negative showed *ErbB2* amplification in their CTCs. However, other studies have shown that *ErbB2* status was similar between breast cancer metastases and the primary tumor (Tanner et al. 2001; Dirix et al. 2005). In this scenario, one can think that disseminated circulating cells may come from subclones of tumor cells and may not be a representative sample of the whole tumor.

Despite these caveats, which warrant further studies, the presence of *ErbB2/TOP2A*-amplified CTCs could serve as a surrogate marker to monitor targeted therapy. A rapid drop in the number of CTCs and cytokeratin-19 mRNA levels has been described in patients after herceptin treatment (Bozionellou et al. 2004). It is likely that the monitoring of the number of cells carrying the specific target against which drugs were designed (whether is herceptin or anthracyclines) would be suitable for testing the efficacy of such therapy. Although we describe here a FICTION method for the analysis of *ErbB2/TOP2A/CEP17* status in TCs from PB, many other breast cancer targets could be analyzed using a similar technology. For instance, a recent paper showed that node-negative breast cancer patients with 11q deletion might benefit from anthracycline-based chemotherapy (Climent et al. 2007).

In summary, we showed the feasibility of a new methodology that combines immunomagnetic selection and FICTION for ErbB2/TOP2A/CEP17, which could allow genetic characterization of CTCs from PB.

Acknowledgments

This study was supported by the "Fundación de Investigación Médica Mutua Madrileña" (to JJG) and partially funded by an ISCIII-RETIC RD06/0020 grant (to AC).

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The Journal of Histochemistry & Cytochemistry

El Escualeno, componente minoritario del aceite de oliva, y su relación con el cáncer

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- Capítulo del libro: *"I Congreso de Cultura del Olivo"*
- Editorial: Instituto de Estudios Giennenses. Diputación Provincial de Jaén
- Fecha de publicación: 2007
- Páginas: 765-782
- Idioma: Español
- ISBN: 978-84-96047-57-0

Instituto de Estudios Giennenses
DIPUTACIÓN PROVINCIAL DE JAÉN

I Congreso de Cultura del Olivo

Edición a cargo de:
Instituto de Estudios Giennenses
DIPUTACIÓN PROVINCIAL DE JAÉN

Diseño y maquetación:
Diputación Provincial de Jaén / Cultura y Deportes

Edita:
Instituto de Estudios Giennenses / Diputación Provincial de Jaén
I.S.B.N. 978-84-96047-57-0

Depósito Legal: J. 433 - 2007

Impresión:
SOPROARGRA, S.A.
Polígono Industrial «Los Olivares»
Villatorres, 10 - 23009 Jaén

Encuadernación:
Olmedo Hermanos, S.L. / Granada

El Escualeno, componente minoritario del aceite de oliva, y su relación con el cáncer

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RESUMEN:

El cáncer es actualmente una de las principales causas de defunción en los países desarrollados. La principal terapia empleada en su lucha, la quimioterapia, es responsable de numerosos efectos secundarios, relacionados en gran medida con la producción de radicales libres y con los efectos que éstos generan en nuestro organismo. Determinados componentes presentes en la naturaleza, poseen propiedades antioxidantes capaces de reducir ese efecto malicioso y de inhibir o frenar el crecimiento de células tumorales in vitro, así como de potenciar el efecto citotóxico de los quimioterápicos. Algunos de los componentes minoritarios presentes en el aceite de oliva presentan características antioxidantes y anticancerígenas, lo que nos ha animado a estudiar estas propiedades en tres de los principales componentes minoritarios del aceite de oliva; el escualeno, el hidroxitirosol y el tirosol.

Aunque el estudio está en una fase temprana y quedan aun numerosos ensayos por realizar, podemos sugerir con los resultados obtenidos hasta la

ABSTRACT:

Cancer is nowadays one of the main causes of decease in developed countries. The most commonly used therapy, the chemotherapy, is responsible for a great number of secondary effects, related with the production of reactive oxygen species and also related with the effects that these reactive oxygen species have on our organism. Specific components present in Nature have antioxidant characteristics, with can reduce this harmful effect and inhibit tumoral cell growth in vitro, as well as potentiate the cytotoxic effects of chemotherapics. Some of the minoritary components presents in olive oil have antioxidant and antitumoral characteristics, fact that encourage us to study these properties in three of the main minoritary components of olive oil; Squalene, Tyrosol and Hydroxytyrosol.

Although the study is in an early stage and there are still numerous assays to make, we can suggest, with the recently obtained results, that the Squalene hasn't got cytotoxic or cytostatic capacity by itself in MDA-MB-231, and

Preliminary study on efficacy and tolerance of a «coupage» of olive oil in patients with chronic kidney disease. Nutritional evaluation [Estudio preliminar sobre eficacia y tolerancia de un «coupage» de aceite de oliva en pacientes con enfermedad renal crónica. Evaluación del estado de nutrición.

Perez Banasco, V., Gil-Cunquero, J.M., Borrego, F.J., Grasso, M., Segura, P., Warleta, F., Lozano, J.L., Costa, L.A., Torres, J., Gaforio, J.J. & Villarrubia, V.G. 2007,

- Trabajo enviado a la revista "*Nefrología*".
- Año de publicación: 2007
- Nº de citas (a 1 de Diciembre de 2015): 3
- SCImago Journal & Country Rank
  - Categoría perteneciente: Nephrology
  - Índice SJR: 0,429
  - H index: 22
  - Posición: 34/59
  - Cuartil en la categoría: Q3
- Tomson Reuters Journal Citation Reports
  - Categoría perteneciente: Urology & Nephrology
  - Índice de impacto: 1,223
  - Posición: 57/78
  - Cuartil en la categoría: Q3

NEFROLOGÍA. Volumen 27. Número 4. 2007

Preliminary study on efficacy and tolerance of a «coupage» of olive oil in patients with chronic kidney disease. Nutritional status assessment

V. Pérez Bañasco, J. M. Gil-Cunquero, F. J. Borrego, M. Grassó, P. Segura, F. Warletta, J. L. Lozano, L. A. Costa, J. Torres, J. J. Gaforio y V. G. Villarrubia

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SUMMARY

The discrepancies among data reported by using olive oil (OO) in humans appear to be due to the great differences between the different OO used. Based on structure/function relationships we have chemically optimized an OO through the rational mixture («coupage») of several Spanish extra virgin olive oils (methodology «oHo»®). Patients with chronic kidney disease (CKD) develop a progressive picture of malnutrition and inflammation that lead them to an elevated risk of cardiovascular disease. In a pilot, randomised trial the nutritional efficacy and safety of «oHo» were evaluated in 32 patients (mean age 60,8 ± 13,2 years old; 16 women) with CKD (KDIGO stages 4-5) at predialysis. After a 7 days wash out for statins and ACE inhibitors 19 patients had «oHo» at doses of 60 mL/day (20 mL t.i.d) for 30 consecutive days, whilst 13 patients remain as a control group without «oHo». At the end of the study only patients having «oHo» showed significant increases of serum albumin ($p < 0.05$) and not significant increases of total proteins, weight, and BMI. Total cholesterol ($p < 0.05$) and HDL-cholesterol ($p < 0.01$) increased with «oHo». The number of cases with pathologic HOMA-IR in the control group increased from 1 to 2 patients whilst in the «oHo» group decreased from 2 to none. No significant changes of minerals, arterial pressure, hemoglobin, and other parameters related to CKD were seen. After a 30 days follow-up in the «oHo» group all parameters came back to basal ones, excepting for blood pressure that significantly decreased ($p < 0,05$). Tolerance was excellent and constipation significantly diminished ($p < 0,001$) in the «oHo» group. Of importance, none of these biological changes were seen in regular consumers of other conventional olive oils (control group). These intriguing results, seen by the first time, appear to partially satisfy the recent claims («reverse epidemiology») about the need of a more correct nutrition in CKD patients. However, these data need to be proved in more larger trials as well as in CKD patients under dialysis with harder inflammatory/malnutrition conditions.

Key words: ***Olive oil. «oHo». Chronic kidney disease. Malnutrition. Inflamation. HOMA.***

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ESTUDIO PRELIMINAR SOBRE EFICACIA Y TOLERANCIA DE UN «COUPAGE» DE ACEITE DE OLIVA EN PACIENTES CON ENFERMEDAD RENAL CRÓNICA. EVALUACIÓN DEL ESTADO DE NUTRICIÓN

RESUMEN

Las discrepancias en las acciones del aceite de oliva (AO) en humanos, parecen deberse a las diferencias existentes entre los distintos aceites utilizados en los estudios publicados, fundamentalmente en sujetos sanos. Basados en relaciones estructura/función, se ha optimizado químicamente un AO mediante la mezcla racional («coupage») de diversos aceites de oliva virgen extra españoles (metodología «oHo»®). Los pacientes con enfermedad renal crónica (ERC) desarrollan un cuadro progresivo de malnutrición e inflamación sobre el que asienta su elevado riesgo de enfermedad cardiovascular. En un estudio piloto, controlado y aleatorizado se ha evaluado la eficacia y seguridad de «oHo» en 32 pacientes (16 mujeres) con ERC (estadios 4-5) en prediálisis, (edad media 60,8 ± 13,2 años). Tras un período de lavado de 7 días para inhibidores de la ECA y estatinas, 19 pacientes tomaron «oHo» (60 ml/día, en 3 tomas) durante 30 días consecutivos y 13 permanecieron como grupo control. Al final del estudio, solamente los pacientes con «oHo» mostraron incrementos significativos en los niveles de albúmina sérica ($p < 0,05$), así como tendencia al aumento del peso y de las proteínas totales. Las cifras de colesterol total ($p < 0,05$) y HDL ($p < 0,01$) aumentaron en el grupo «oHo». El número de casos con HOMA patológico subió de 1 a 2 pacientes en el control, mientras que en el grupo «oHo» los 2 pacientes iniciales con HOMA patológico normalizaron sus índices. No se observaron cambios en los parámetros relacionados específicamente con la ERC: minerales, creatinina, anemia, etc. Tras un período de seguimiento de 30 días, todos los parámetros que cambiaron en el tratamiento regresaron a cifras basales, excepto la presión arterial media, que disminuyó ($p < 0,05$). La tolerancia fue excelente y el estreñimiento disminuyó significativamente (< 0,001) en el grupo «oHo». Dada la originalidad del estudio, estos resultados deberán ser comprobados con estudios más amplios.

Palabras clave: ***Aceite de oliva. «Coupage oHo». Enfermedad renal crónica. Malnutrición. Inflamación. HOMA.***

INTRODUCTION

Nutritional status is a key factor in the clinical course of patients with chronic renal disease (CRD).[1] Patients with CRD have high prevalence of malnourishment that presents æ from the beginning of the diseaseæ due to low levels of serum proteins together with weight loss, both conditions being related with increased morbidity and mortality rated.[1-3]

It is currently believed that malnourishment, partially due to uremia-induced anorexia, is not the cause of these changes and that muscle mass loss is due to endogenous proteolytic mechanisms.[4] In fact, it seems that hypoalbuminemia is not as much due to poor protein intake as to the presence of pathological conditions that promote these mechanisms, among which inflammation,[5] metabolic acidosis,[6] as well as other CRD-related pathologic conditions, such as insulin resistance (IR)[4] stand out. In any case, hypoalbuminemia is currently considered as: a) a cause and/or main consequence of malnourishment and inflammation in these patients; b) a known predicting factor of cardiovascular complications and of morbid-mortality increase in CRD patients, including those that have not started yet on dialysis.[1,7-9]

For all this, it is believed that correct dietary intake with several supplements, either orally or through intra-dialytic parenteral nutrition (IPN) with

essential amino acids, may be essential in the course of CRD.[3,10-12] More recently, the use of lipid emulsions based on soy and/or olive oils for IPN administration represents a new tool in nutritional management of CRD patients. Thus, it has been described that these emulsions to alleviate particular protein deficiencies including recovering of albumin serum levels.[13]

This rationale, together with the high costs associated with IPN,[10] led us to try to recover the nutritional status of our patients by means of olive oil (OO). It is today clear, however, that several of the beneficial actions ascribed to olive oil in humans are paradoxical and seem to depend on the type of OO used in each study as well as the pathological condition considered. In this way, although there is some consensus on the beneficial effects of virgin OO in patients with cardiovascular risk,[14] other contradictory effects on HDL-cholesterol[15-22] and on the IR phenomenon[20,23-29] in other pathologies have been described.

Strangely, a study on CRD patients reports on the lack of effect of OO on total cholesterol and HDL-cholesterol levels.[22]

Aside from these paradoxical actions of the different OO, it has not been considered either that some of them may contain substances interfering with their expected biological actions. Thus, pesticides and other synthetic chemical compounds behave as confounding factors for the endocrine system.[30] Since their presence in OO is often times higher than that contained in olives themselves,[31] it should be tried that olive oils used for patients are devoid of these substances. In the case we are dealing with, the reasonable doubt of their possible implication in the pathogenesis of several inflammatory diseases, including CRD itself, is emerging.[32-34]

For these reasons, together with other derived from several genetic and agrologic conditions inherent to the olive tree, it was decided to perform a first rational blend («coupage») of different extra organic/ecologic virgin OO in order to optimize their functional properties in humans.[35] This first organic «oHo» coupage is assessed in this nutritional study in patients with advanced CRD that will be completed with other ongoing immunological studies.

PATIENTS, MATERIAL AND METHODS

Patients: 32 patients (16 women), mean age 60.8 ± 12.8 years, diagnosed with CRD stages 4-5 of the KDIGO classification.[36] Other demographic and clinical characteristics are summarized in Table I. Patients were followed at the Pre-dialysis clinic of the Nephrology Department of the University Hospital of Jaen.

Inclusion criteria: Non-diabetic patients of both genders, older than 75 years, with advanced CRD that had not started on dialysis. They had not to present previous history of any disease that could interfere with the nutritional and inflammatory status of CRD, such as cancer, AIDS, hepatitis, tuberculosis, lupus, or rheumatoid arthritis, among others. During the 7 days prior to inclusion into the study (wash-out period), patients did not received any drugs that may affect the inflammatory reactivity such as steroids, ACE inhibitors (ACEI) or statins, although they may receive other conventional antihypertensive in case of necessary. All patients signed their informed consent. The study was approved by the Hospital's Ethic Committee.

Exclusion criteria: excluded patients were those with diabetes mellitus, congenital or acquired immunodeficiencies, with kidney or other grafts, obesity with BMI > 40 kg/m^2 and also those having received anti-inflammatory medications within 7 days prior to inclusion into the study.

«Coupage oHo» of organic olive oil: this is a rational blend (Methodology «oHo»®) from different extra virgin Spanish olive oils, which is based on a previous and comprehensive analysis of the qualitative and quantitative chemical composition of each oil used. Based on the known structural/functional relationships, the aim is to harmonize the presence of the components needed to do a particular biological function, in this case nutritional and anti-oxidant [the latter will be the subject of another publication]. All OO used came from organic (ecological) crops, endorsed by ecological accreditations (ACEA: Andalusian Committee of Ecological Agriculture), the final product being classified as extra virgin olive oil (Table II).[37] «oHo» was delivered bottled in dark glass labeled 500-mL bottles together with a dosing syringe and a recording log of the daily dose consumed by the patient. Each label contained the study number, the patient's code and initials as well as the date of delivery and recommendations for oil preservation and administration.

Analytical parameters: *a) Of efficacy*: the parameters analyzed were those directly related with the nutritional status (Table III), such as weight and body mass index (BMI), serum protein levels, lipid profile, glucose profile, as well as other indirect parameters

Table I. Demographical characteristics and CRD-related parameters

Characteristics	Study Groups: Control (w/o «oHo» oil)	Study Groups: «oHo»
Total patients (%)	13 (100%)	19 (100%)
Gender (M/F)	7/6	9/10
Age (years): X ± SD	61.4 ± 16.4	60.3 ± 10.1
• Women	59.5 ± 20.5	60.9 ± 10
	13 (100)	19 (100)
Usual OO consumers		
CRD parameters:		
• Creatinine clearance (mL/min)	18.9 ± 8.2	17.8 ± 7
• Urea clearance (mL/min)	8.5 ± 3.4	7.9 ± 2
• Plasma creatinine (mg/dL)	4.1 ± 2.1	4.8 ± 1.8
• Plasma urea (mg/dL)	133 ± 51	150 ± 42
	4 (30.7)	7 (36.8)
Num. and (%) of patientes in stage 5*		

OO: conventional olive oil; *According to the KDIGO classification[72]. No significant differences were found between groups.

Table II. Main components in «oHo»

Components	Values
FATTY ACIDS (%):	77.91
• **Mono unsaturated (MUFA):**	0.91
☐ Oleic	5.67
☐ Palmitolic	0.55
• **Poly unsaturated (PUFA):**	11.5
☐ Linoleic	2.85
☐ Linolenic	
• **Saturated (SFA):**	
☐ Palmitic	
☐ Stearic	
Total PHYTOSTEROLS (mg/kg)	1197
• Beta-sitosterol (%)	95.2
• Campesterol (%)	3
POLY PHENOLS (mg/kg)	10.93
• Hydroxytyrosol	0.01
• Oleuropein	0.63
• Vanillin	0.07
• Ferulic	
OTHER (mg/kg):	160.5
• Alfa- tocopherol (vitamin E)	1.5
• Beta-carotene (pro-vitamin A)	
FATS *trans* (*t*)	0.02 %
• Oleic *t* isomers	0.02 %
• Linoleic *t* + *t* Isomers	
WAXES (mg/kg)	38
PROTEINS	0
CARBOHYDRATES	0
PESTICIDES AND HERBICIDES	Not detected

«oHo» is an extra virgin olive oil, according to EEC Regulation 2568/91 and further modifications on vegetal fat consumption.[37]

(serum transaminase and homocysteine levels); ***b) of tolerability***: parameters related with the clinical situation of CRD (Table IV).

All the parameters were measured at the Clinical Laboratory Department of our hospital by using conventional diagnostic methods. Total proteins, albumin and homocysteine were assessed by nephelometry (Dade, Behring, Germany). Creatinine, urea, glucose, total triglycerides and HDL were deter-

Table III. Effects of «oHo» consumption for 30 days on nutritional parameters of CRD patients. Comparison with the Control Group that did not receive this olive oil

Nutritional Parameters	Assessment points / groups: Baseline (Day 0) Control n = 13	Baseline (Day 0) «oHo» n = 19	Final (Day +30) Control n = 13	Final (Day +30) «oHo» n = 19
NUTRITIONAL PROFILE				
Weight (kg)	66.6 ± 12.9	73.6 ± 10	66.5 ± 13	74 ± 10.8
Body mass index (kg/m^2)	27.9 ± 3.2	28.4 ± 4.6	27.8 ± 3.2	28.6 ± 4.8
Total proteins (g/dL)	6.95 ± 0.5	6.9 ± 1.3	6.9 ± 0.5	7.4 ± 0.5#
Albumin (g/dL)	4.1 ± 0.3	4.1 ± 0.8	4.0 ± 0.3	4.5 ± 0.4*
LIPID PROFILE				
Triglycerides (mg/dL)	112 ± 70.2	129 ± 62.7	115 ± 65.1	132 ± 51.4
Total cholesterol (mg/dL)	187 ± 44.2	192 ± 59.5	188 ± 50.7	214 ± 51.1*
LDL-cholesterol (mg/dL)	115 ± 28.9	114 ± 43.9	115 ± 38	124.3 ± 40
HDL-cholesterol (mg/dL)	49 ± 11	53 ± 16.3	50 ± 11.5	63 ± 20.9**
GLYCEMIC PROFILE				
Glucose (mg/dL)	89.2 ± 9.6	91 ± 14.7	88.5 ± 11.7	94.3 ± 8.6
Insulin (mU/mL)	9.4 ± 6.2	8.2 ± 3.4	10.5 ± 5.8	8 ± 2.7
HOMA index	2.1 ± 1.4	1.9 ± 0.97	2.2 ± 1.3	1.9 ± 0.69
Num. and (%) of cases with pathological HOMA index*	1 (7.7)	2 (10.5)	2 (15.4)	0 (0) [1 (5)]

N: number of evaluated cases; At baseline there were no significant differences between the study groups; # P = 0.056. * P < 0.05 and ** P < 0.01 vs. baseline and Control Group. The pathological HOMA index is defined here as ^{3}3,9 en women and ^{3}3,5 values in men. The figure between brackets represents the number of patients with pathological HOMA index during the follow-up period (day +60) in the «oHo» Group.

Table IV. Effects of «oHo» consumption for 30 days on directly CRD-dependent variables

Assessment points / Groups				
Parameters	**Baseline (Day 0)**		**Final (Day+ 30)**	
	Control n = 13	**«oHo» n = 19**	**Control n = 13**	**«oHo» n = 19**
Hemoglobin (g/dL)	12.3 ± 1.5	12.2 ± 0.8	12.3 ± 1.7	12.3 ± 0.8
Calcium (mg/dL)	9.3 ± 0.6	9.7 ± 1.4	9.3 ± 0.6	10.2 ± 0.6
Phosphorus (mg/dL)	4.2 ± 1.3	4.8 ± 1.3	4.2 ± 1.1	4.7 ± 1.2
Bicarbonate (mEq/L)	19.8 ± 3.7	21.4 ± 2.2	20.4 ± 3.8	20.9 ± 1.7
Urea (mg/dL)	133 ± 51	150 ± 42	138 ± 65	153 ± 39
Urea clearance (mL/minute)	8.5 ± 3.4	7.9 ± 2	8.06 ± 4.1	7.06 ± 2.5* [7.3 ± 2.7]
Plasma Cr. (mg/dL)	4.1 ± 2.1	4.8 ± 1.8	4.3 ± 2.2	5.1 ± 1.6
Cr. Clearance (mL/minute)	18.9 ± 8.2	17.8 ± 7	19.4 ± 10.3	14.7 ± 6* [16.7 ± 6.7]
intact PTH (pg/mL)	181 ± 134	216 ± 246	176 ± 85	256 ± 191
GOT (UI/L)	21 ± 10.3	16.5 ± 5.2	17.7 ± 6.9	19.4 ± 4.7* [16.3 ± 4.2]

Cr: Creatinine; N: number of cases evaluated. At baseline there were no significant differences between the study groups; * P < 0.05 vs. baseline. * P < 0.05 vs. baseline. The figures between brackets represent the values obtained during the follow-up period (day +60).

mined by enzymatic colorimetric methods adapted to an automatic Olympus 5400 analyzer. LDL-cholesterol was calculated by the Friedewald's formula. An ADVIA 120 counter (Bayer Diagnostic SL) was use for hemoglobin. Plasma insulin levels were determined by enzyme immunoanalysis (Axin, Abbott) and PTH by chemiluminescent method with a DXI 800 analyzer (Izasa SA). The HOMA-IR formula was used to calculate the insulin resistance index = [insulin (mU/mL) x glucose (mmol/L)]/22.5, where a high index reflects low sensibility to insulin.[38] A pathological HOMA was defined as ≥3,9 in women and ≥3,5 in men. Creatinine clearances and urea were calculated by the general clearance calculation (vol. min x urine parameter/blood parameter).

Other safety and tolerability clinical parameters

At the end of the period of oil consumption (day +30), patients were asked about the level of acceptance of «oHo» consumption, as well as the comfort or discomfort feeling experienced. Similarly, the number of cases having intestinal constipation at the beginning and at the end of the study was registered by means of a semi-quantitative question/answer scale. Constipation was considered to be present if: a) defecation was every two or more days; b) feces were of hard consistency; and c) there was discomfort when defecating.

Methods

During the month before entry into the study, patients were randomly assigned by order of arrival to consultation (odd or even order) to one of two study groups (Table I): Control Group, comprised by 13 patients (odd order of arrival) that did not received «oHo», and the «oHo» Group comprised by 19 patients (even order of arrival) that received «oHo». At day–1, each patient of the «oHo» Group received 4 bottles (2 L) of oil, a sufficient amount for the 30 study days. Each patient had to take 60 mL of «oHo»/day, distributed in three doses taken at breakfast, lunch, and dinner. The oil had to be always taken crude, directly taken or applied to natural foods from the diet, e.g., bread. During the study, patients were forbidden to take any other crude oil although they could still use their usual oils for cooking. We may point out that in our region (Jaen) it is routine practice in the general population to use olive oil, so that a especial emphasis was put on these recommendations (Table I). At baseline (day 0) and end of the study (day +30), all the clinical and laboratory parameters described were assessed.

Follow-up period

The group taking «oHo» was closely monitored throughout the 30 days following the end of olive oil consumption. At the end of this follow-up period (day +60 after the study beginning), all the panel of laboratory work-up above-mentioned was repeated in order to compare the control group and the «oHo» group.

Statistical analysis

For quantitative variables, the mean, median, standard deviation and maximal and minimal values were calculated. The sample was grouped according to the study group and the different variables measured at days 0, +30, and +60 were compared by non-parametric tests for related samples. Then, the incremental value between days 0 and +30 for the

different variables were calculated and the differences between the Control and «oHo» groups were compared by the Mann-Whitney test for independent samples. The McNemar' s test was used to compare the proportions in related samples. For those variables not following a normal distribution, such as PTH or alkaline phosphatase, a logarithmic transformation was used. The statistical analysis was done by means of the SPSS® statistical package for Windows, version 11.0.

RESULTS

Initial design and withdrawals from the study

This pilot study was designed aiming at studying the immunological changes produced by «oHo» consumption and, based on that, the sample size was calculated. Only 32 out of 40 (20 per group) expected patients strictly met the inclusion and exclusion criteria. During the first 15 study days, there were two withdrawals (one in the «oHo» Group and another one in the Control Group), because of having during this period continuous worsening of previous renal symptomatology that led to starting on dialysis. Three patients in the Control Group voluntarily withdrew from the study due to commuting problems, and three other withdrew at day–1 without giving any explanation. Due to the especial inclusion and exclusion characteristics, together with the need for blood samples in order to study the ongoing immunological changes (Second part of the study), these patients could not be replaced by other ones having similar characteristics to those initially recruited.

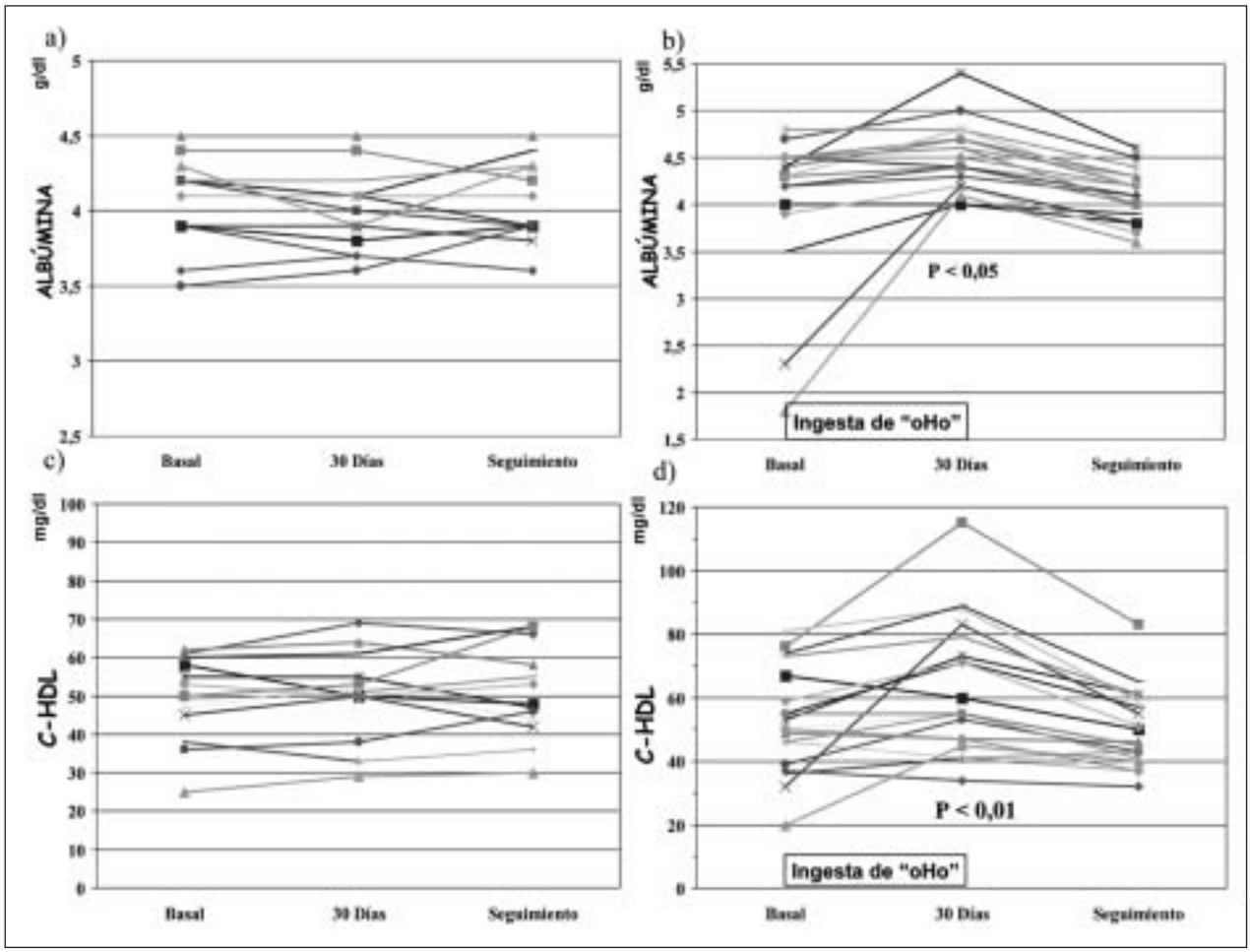


Fig. 1.—Progression of serum albumin (a and b) and HDL-cholesterol (c and d) levels observed during the study. Comparison between the Control Group (a and c) and that receiving «oHo» (b and d).

Characteristics of the patients finally included

Table I shows the demographical and CRD characteristics of the study population. At baseline, there were no significant differences in any of the parameters shown on the Table.

Nutritional status: profiles of proteins, lipids, and sugars. HOMA Index

During «oHo» consumption (Table III), no significant increases in weight and BMI were observed, as well as levels of plasma proteins ($p = 0.056$). These increases were significant for albumin ($p < 0.05$). In the Control Group, these parameters slightly decreased without reaching statistical significance (Table III). At day +60 (follow-up period in the «oHo» Group), all the nutritional parameters decreased (data not shown), returning to values similar to baseline ones. For instance, Figs. 1a and 1b show the progression of albumin during all the study period for both groups.

Triglycerides levels increased in a non-significant manner in both groups (Table III). Total cholesterol significantly increased ($p < 0.05$) in the «oHo» Group. LDL-cholesterol plasma levels did not change in any of the groups. HDL-cholesterol levels significantly increased ($p < 0.01$) only in the «oHo» group. During the follow-up period, triglycerides and LDL levels increased in a non-significant manner in both groups (data not shown), whereas HDL-cholesterol levels decreased in the «oHo» Group down to not showing significant differences with the baseline period (Figs. 1c and 1d).

There were no significant changes in sugar profile (Table III). In the Control Group one patient showed a pathological HOMA index at baseline, and this number increased to two patients throughout the study, whereas the two patients showing a pathological HOMA index at baseline in the «oHo» Group returned to normal (Table III). At the end of the follow-up period, one patient in the «oHo» Group presented once again a pathological HOMA index (Table III).

Minerals and other CRD-related parameters

In the «oHo» Group (Table IV), significant ($p < 0.05$) descents in urea and creatinine (without associated symptoms and with no need for changes in «oHo» administration), and not significant descents in phosphorus and bicarbonate levels were observed. Fig. 2a represents how the discontinuation of «oHo» consumption (follow-up, day +60) produced a return to baseline values.

Tolerability and safety parameters

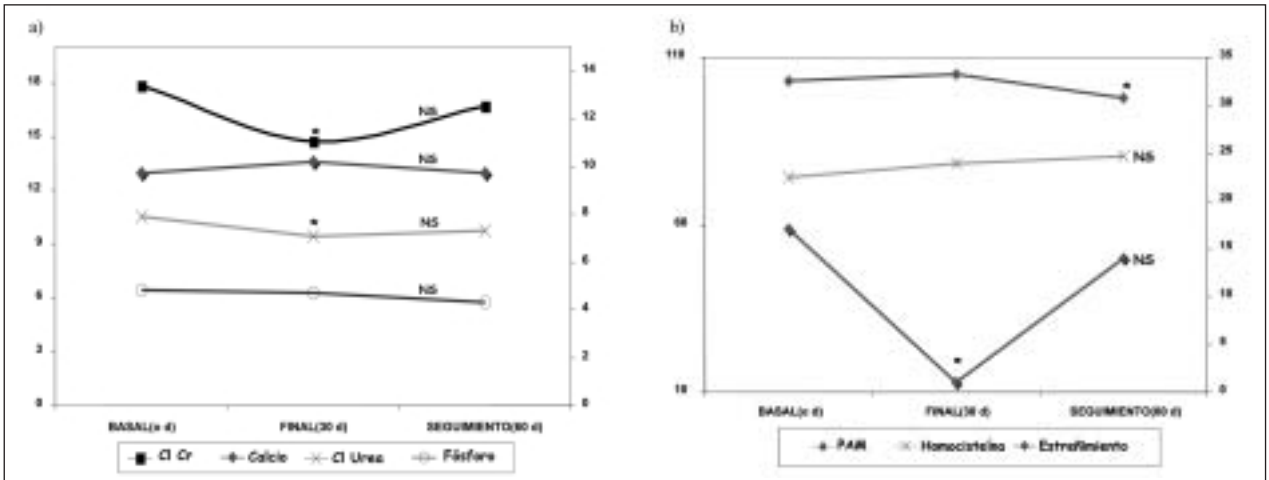
Between the baseline (day 0) and final (day +30) periods there were no changes in any group of the values for mean blood pressure, transaminase, GPT, and homocysteine (data not shown). The GOT values significantly increased ($p < 0.05$) in the «oHo» Group although it came back to during the follow-up period (Table IV). No changes regarding constipation were observed in the Control Group, whereas in the «oHo» Group this parameter significantly decreased during «oHo» consumption ($p < 0.001$) (Fig. 2b). Assessment at follow-up (day +60) in the «oHo» Group showed a return to baseline values of transaminase (GOT and GPT) and PTH levels, whereas homocysteine levels increased ($p < 0.05$) (Fig. 2b). The percentage of constipated patients returned to baseline values (Fig. 2b) and mean blood pressure (MBP) significantly decreased during the follow-up period ($p < 0.01$) as compared to baseline (Fig. 2b). Acceptance of «oHo» was excellent in all cases and no intake-related side effects were observed.

DISCUSSION

This is the first study aiming at verifying the nutritional effects of an olive oil in CRD patients. The results obtained indicate that nutritional supplement with and organic extra virgin «oHo» olive oil improves homeostasis within the protein, lipid, and glycidic compartments, while maintaining an adequate energy load and without side effects in patients with advanced CRD, stages 4-5. These effects fade upon discontinuing «oHo» consumption.

With regards to the protein compartment (Table III), the improvement in the nutritional status produced by «oHo» is represented by significant increased in weight (average of 400 g), BMI total plasma proteins levels; these increases might reach statistical significance by increasing the number of patients or the product administration time. In any case, 30 days of «oHo» administration were enough to significantly increase plasma albumin levels.

The increased in albumin observed are similar to those obtained with the use of other oral complex supplements [containing fat, proteins, anti-oxidant and anti-inflammatory agents][12] or olive and/or soy oil-containing emulsions used in IPN.[13,39-41] Whereas the methods cited base part of their composition in essential amino acids, it is evident that «oHo»



CrCl: creatinine clearance; UrCl: urea clearance. * P < 0.05 and ** P < 0.001 vs. baseline; NS: not significant as compared to baseline.

Fig. 2.—Progression of urea and creatinine clearances and calcium and phosphorus levels (2a) and values referred for mean blood pressure, homocysteine and constipation (2b) observed during the study period in the «oHo» group.

does not contain any protein material (Table II), clearly indicating that the mechanism of protein recovery observed in this study is not based on direct protein or protein-precursors consumption. Fortunately, an spite of the notable increase of protein nutritional status, the (not significant) increases in blood urea levels in the «oHo» Group were slightly lower to those observed in the Control Group (Table IV), indicating that consumption of the test product does not interfere with catabolic processes leading to increased urea production in CRD patients. Considering the close relationship between uremia and hypoalbuminemia,[6] these actions of the «oHo» could also contribute to nutritional status improvement. This improvement is achieved within 30 days, which is in contrast with the need for longer therapies, 3-12 months long, with IPN,[39-41] except for one study, and fades when the product is discontinued (Fig. 1b). This fact is the opposite to what has been observed in the Control Group (Fig. 1a), thus corroborating the truth of the described effects.

The impact of the nutritional effects of «oHo» on the inflammatory status of CRD patients are, until now, unknown, although there is a consensus on the close relationship between hypoalbuminemia and inflammation in these patients,[4,5,8,9,12] which already suggests the possible beneficial effect of «oHo».

About the lipidic compartment (Table III), improvement of the nutritional status was shown by significant increases in total cholesterol, mainly due to significant increases in HDL-cholesterol levels. These beneficial effects of «oHo» on the lipid profile are opposite to what has been observed with the use of olive oil emulsions for IPN, where total plasma fat content decreased by 50% and was accompanied by significant increases in LDL levels and significant decreases in HDL-cholesterol levels.[13] Other studies with soy oil emulsions in IPN[39] or with emulsions free from amino acids supplements[41] do not show either any effect on lipidic plasma composition in hemodialyzed patients. Similarly, although the effects of conventional OO on HDL-cholesterol levels in CRD patients are controversial,[15-21] one trial describes the lack of an effect of OO on total cholesterol and HDL-cholesterol levels in the CRD pathologic condition.[22]

The reality of these effects of «oHo» on HDL-cholesterol is represented in Fig. 1d that shows how the discontinuation of the product led to a decrease in HDL-cholesterol levels, by contrast to what happened in the Control Group (Fig. 1c). Consequently, and given the know cardiovascular protective effect ascribed to HDL in CRD patients,[42] the effects of «oHo» on this parameter should be regarded within its probable cardiovascular preventive dimension.

About sugars metabolism, an anecdotic decrease in the number of patients presented a pathological HOMA index at the study beginning, accompanied by a relapse during the follow-up period (Table III), suggests the positive effects of the product on IR. Given that the actions of conventional OO on HOMA-IR are controversial,[20,23-29] we will have to

wait until larger studies can clarify this situation.

In summary, taking into account that hypoalbuminemia, low HDL levels, and insulin resistance are potent predicting factors of the cardiovascular risk, morbidity and mortality in CRD patients,[1-13,42] the effects here described with «oHo» bring a new preventive and/or therapeutic alternative for these patients, always with appropriate caution.

The tolerability was excellent and no unwanted clinical side effects related with the product were observed. Consumption of «oHo» for 30 days did not change hemoglobin levels, the mineral profile, or urea and creatinine plasma levels (in spite of an increase in plasma proteins), although significant descents in urea and creatinine clearances were observed at the end of the administration period (Table IV). Regarding these latter functional changes, it seems clear that: 1) even from baseline, and just because of the random effect, the number of stage 5 CRD patients was higher in the «oHo» Group (Tables I and IV); 2) the decrease in clearances was not accompanied by other changes, such as decreased diuresis, increased acidosis, or increased hyperkalemia (data not shown); 3) these values returned to baseline values when «oHo» was discontinued (Fig. 2a); 4) nephrotoxic changes have never been described in relation with OO ingestion. It is evident that, given the state of advanced renal failure, these patients have no margins of physiological adaptation before the overload intake related with «oHo». Anyhow, the existence of these facts should be taken into account and verified in larger studies, especially in pre-dialysis patients.

Three other facts stand out in this preliminary study: 1) the dramatic decrease in the percentage of constipated patients during «oHo» consumption is a robust fact (Fig. 2b), which with no doubt has an impact on daily quality of life of CRD patients; 2) phosphorus values did not change or had a tendency to decrease (Fig. 2a), in spite of the nutritional status improvement; and 3) the behavior of mean blood pressure is also striking, which although it did not vary during «oHo» consumption, it significantly decreased during the follow-up period (Fig. 2b). Although we have not an explanation for this fact, it seems clear that conventional OO does not reduce blood pressure in CRD patients[22] under specific antihypertensive therapies.

Finally, we should point out the nutritional requirements of CRD patients that sometimes are very different from those of healthy individuals. Thus, the paradox claiming that, by contrast to what happens in healthy individuals («reverse epidemiology»), the poorer vital prognosis in CRD patients is directly related with low values of BMI, blood pressure and serum cholesterol, homocysteine, and creatinine levels, sets the need for interventional studies aiming at corroborating whether weight gain in CRD patients may increase their survival and improve their quality of life.[1] Besides, the ongoing immunological studies ought to clarify the unknown issues related with the nutritional status and the effects of «oHo».

ACKNOWLEDGMENTS

We would like to thank the dedication and professionalism of the nurses Francisca Muelas Ortega and Sagrario Jiménez Jiménez, and the great and altruistic work of the study coordinator, María Teresa Jódar Mejía (R+D+i of Bioaveda).

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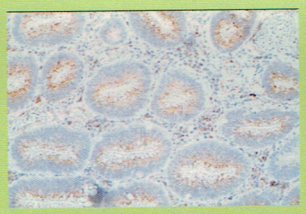
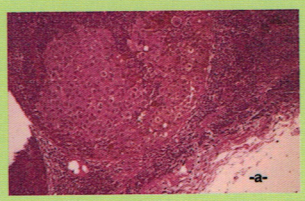
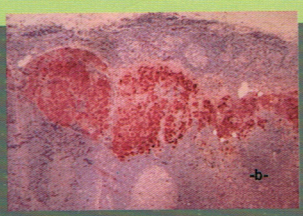
Warleta, F., Ruiz-Mora, J., Algarra, I. & Gaforio, J.J. 2006,

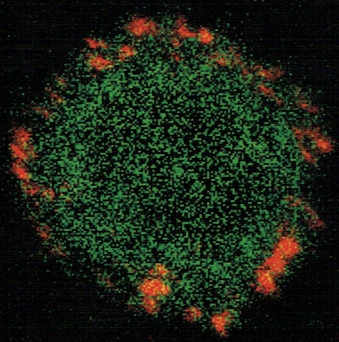
- Capítulo del libro: "*Avances en Oncología Básica y Aplicada"*
- Editorial: Universidad de Granada
- Editado por: J.A. Marchal, J.J. Gaforio & A. Aránega
- Fecha de publicación: 2006
- Páginas: 505-534
- Idioma: Español
- ISBN: 84-338-3907-1

Juan Antonio MARCHAL CORRALES
Jose Juan GAFORIO MARTÍNEZ
Antonia ARÁNEGA JIMÉNEZ









Avances en Oncología Básica y Aplicada

JUAN ANTONIO MARCHAL CORRALES
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AVANCES EN ONCOLOGÍA BÁSICA Y APLICADA

GRANADA
2006

AVANCES EN ONCOLOGÍA BÁSICA Y APLICADA
I.S.B.N.: 84–338–3907–1. Depósito legal: GR/1.507–2006.
Edita: Editorial Universidad de Granada.
Campus Universitario de Cartuja. Granada.
Fotocomposición: Portada Fotocomposición, S. L. Granada
Imprime: Imprenta Comercial. Motril. Granada.
Printed in Spain *Impreso en España*

ACEITE DE OLIVA Y CÁNCER. COMPONENTES BIOACTIVOS CON CAPACIDAD ANTITUMORAL

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1. ACEITE DE OLIVA

La palabra aceite deriva del nombre árabe *az-zait,* que significa «jugo de oliva».

En la actualidad, España es el país con más cantidad de olivos superando los 300.000.000, seguido de lejos por Grecia e Italia. Asimismo, es el mayor productor y exportador mundial de aceite de oliva con una producción media que llega a alcanzar 1.000.000 de toneladas al año (en el 2002 fue de 1.300.000 toneladas), y es en Andalucía donde se concentra el 80% de la producción de ese aceite.

Todas las grasas se componen de ésteres de glicerina y ácidos grasos. Entre estos ácidos grasos, los más abundantes en los organismos son el palmítico (tripalmitina), esteárico (triestearina) y oleico (trioleina). Mientras que los dos primeros son sólidos a temperatura ambiente, la trioleina se mantiene líquida a temperaturas relativamente bajas, y esta característica es la que diferencia las grasas sólidas de las grasas líquidas, también conocidas como *«aceites»* (Morros Sardá A y cols., 2000).

Por su composición en glicéridos, podemos clasificar al Aceite de Oliva dentro del grupo de aceites vegetales no secantes, por ser líquido a temperatura ambiente; lo que los diferencia de las grasas que son sólidas a temperatura ambiente, ser de origen vegetal y no endurecerse al exponerse a la luz y al aire (Ver figura 1).

El Aceite de Oliva Virgen Extra es el zumo de la aceituna que, por su obtención exclusivamente mediante la primera prensa en frío, conserva todas sus características físico-químicas inalteradas y, por tanto, presenta los mayores valores de aquellos componentes beneficiosos para la salud como las vitaminas A, D, E y K, clorofila, esteroles, tocoferoles y compuestos fenólicos. Otros aceites, como el de girasol, maíz,

Aceite de Oliva en la Salud y en la Enfermedad

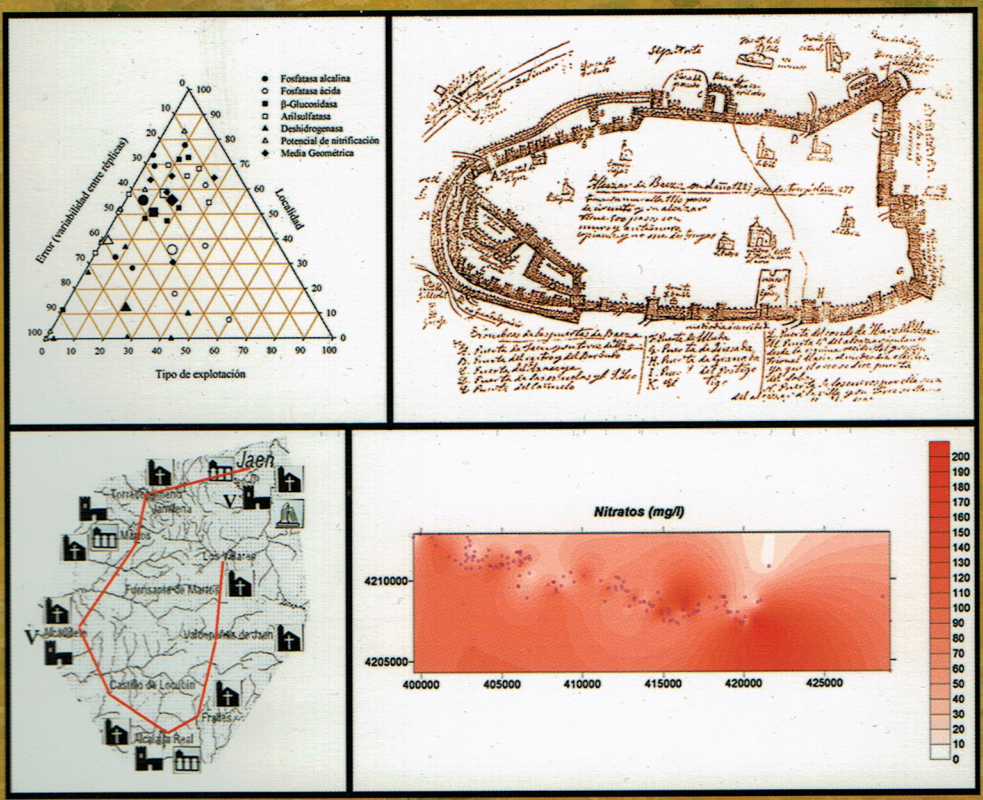
Warleta, F., Ruiz-Mora, J., Campos, M., Algarra, I., y Gaforio, J.J.

- Capítulo del libro: "*Proyectos de Investigación 2003-2004"*
- Editorial: Universidad de Jaén
- Editado por: Antonio Gálvez del Postigo
- Fecha de publicación: 2005
- Páginas: 309-370
- Idioma: Español
- ISBN: 84-8439-271-6

PROYECTOS DE INVESTIGACIÓN

2003 - 2004

Antonio Gálvez del Postigo
(Coordinador)



UNIVERSIDAD DE JAÉN

CAJA RURAL JAEN

ACEITE DE OLIVA EN LA SALUD Y EN LA ENFERMEDAD

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RESUMEN

El aceite de oliva desde tiempos remotos ha sido un elemento esencial para el hombre, de forma que a lo largo de los años se ha ido incrementando el interés por este alimento fundamental de la dieta mediterránea. El cáncer de mama entre otras enfermedades, está adquiriendo un gran protagonismo con unos 16.000 casos nuevos anuales en España. El aceite de oliva gracias a su composición rica en ácido oleico así como otros componentes minoritarios con propiedades antioxidantes y antiinflamatorias, puede jugar un papel importante para ayudar a prevenir o combatir esta enfermedad. En nuestro trabajo pretendemos demostrar si componentes que forman parte de la fracción insaponificable del aceite de oliva virgen extra (Hidroxitirosol, Tirosol y Escualeno), incrementan el efecto apoptótico de determinados quimioterápicos (Paclitaxel, Adriamicina y Gemcitabina) sobre células tumorales de cáncer de mama.

Asimismo, queremos comprobar si los mencionados componentes minoritarios potencian la fagocitosis de células tumorales de mama en apoptosis por parte de macrófagos peritoneales procedentes de ratones BALB/c.

Es por esto por lo que nuestro estudio podría tener una elevada trascendencia, suponiendo un nuevo avance en la terapia frente a esta enfermedad, además de incrementar el interés socio-sanitario que despierta el aceite de oliva prácticamente en todo el mundo.

ABSTRACT

Olive oil has been an essential substance for the human since ever, so through time, there has been an increasing interest in this fundamental ingredient of the mediterranean diet. Breast cancer, among other diseases, is getting a very important role bearing in mind that there are about 16000 new cases per year in Spain. Olive oil, thank to its composition in oleic acid and other minority components with antioxidants and anti-inflammatory properties, could play a very decisive part in this disease. In the present investigation, we pretend to test