UNIVERSIDAD DE JAÉN

FACULTAD CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE CIENCIAS DE LA SALUD

TESIS DOCTORAL

ESTUDIO DE LAS PROPIEDADES ANTITUMORALES, ANTIOXIDANTES Y ANTIINFLAMATORIAS DE LOS PRINCIPALES TRITERPENOS DEL ACEITE DE OLIVA VIRGEN UTILIZANDO MODELOS EXPERIMENTALES CELULARES DE MAMA

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JAÉN, 14 DE NOVIEMBRE DE 2014

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CERTIFICA: Que el trabajo expuesto en la presente Tesis Doctoral:
"Estudio de las propiedades antitumorales, antioxidantes y antiinflamatorias de los principales triterpenos del Aceite de Oliva Virgen utilizando modelos experimentales celulares de mama" presentado por Dª. Cristina Sánchez Quesada 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.

Jaén, lunes, 23 de septiembre de 2014

Cristina Sánchez Quesada   José Juan Gaforio Martínez
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"Produce una inmensa tristeza pensar que la naturaleza habla mientras el género humano no escucha"

Victor Marie Hugo (1802-1885)

NOMENCLATURA

ADN   Ácido desoxirribonucleico

Akt   Protein kinase B (proteína quinasa B)

AOV   Aceite de oliva virgen

AP-1   Activating-protein-1 (proteína de activación 1)

APAF-1   Apoptosis protease-activating factor-1 (Factor activador de la proteasa apoptótica 1)

ARNm   ácido ribonucleico mensajero

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)

Ca^{2+}   Ion calcio

CAT   Catalasa

CD   Cluster of differentiation (cúmulo de diferenciación)

Ced-4   Cell death protein 4 (proteína de muerte celular 4)

C-myc   myelocytomatosis viral oncogene analogue (oncogén análogo al viral de mielocitomatosis)

COX   Cyclo-oxigenase (ciclooxigenasa)

EMT   Epithelial mesenchymal transition (transición epitelio mesénquima)

ER   Eritrodiol

ER-   Estrógeno receptor negativo

ERK1/2   Extracellular signal-regulated kinase (quinasa reguladora por señales extracelulares)

ERRα   Estrogen related receptor alpha (Receptor relacionado con los estrógenos alpha)

EROs   Especies reactivas de oxigeno

Fas   Fas cell Surface death receptor (receptor de muerte celular de membrana)

FSP1   Fibroblast secreted protein-1 (proteína 1 secretada por los fibroblastos)

Nomenclatura

h	horas
HIF	Hypoxia-induced factor (Factor de hipoxia inducido)
H_2O_2	peróxido de hidrógeno
IL	Interleuquina/citoquina
IL-1β	Interlequina 1 beta
INF-γ	Interferón gamma
iNOS	Nitric oxide synthase (óxido nítrico sintasa inducible)
IRF-5	Interferon regulatory factor 5 (factor de regulación del interferón 5)
JNK	Quinasas c-Jun N-terminal
K	Potasio
LDH	Lactate dehydrogenase (lactato deshidrogenasa)
LDL	Low-density lipoprotein (lipoproteína de baja densidad)
LOX-5	5-lypoxygenase (5- lipooxigenasa)
MA	Maslinic acid (Ácido maslínico)
MAPK	Mitogen-activated protein kinase (quinasa proteica activada por mitógeno)
MHC	Major histocompatibility complex (complejo mayor de histocompatibilidad)
min	Minutos
MMPs	Membrane-anchored matrix metalloproteinases (metaloproteasas de la matriz ancladas a la membrana)
mTOR	Mammalian Target of Rapamycin (diana de rapamicina en células de mamífero)
MUFA	Monounsaturated fatty acids (ácidos grasos monoinsaturados)
NF$\kappa\beta$	Nuclear factor kappa beta (factor nuclear kappa beta)
nM	Nanomolar
NO	Óxido nítrico
NRF1	Nuclear respiratory factor 1 (factor nuclear respiratorio 1)
NRF2	Nuclear respiratory factor 2 (factor nuclear respiratorio 2)
OA	Oleanolic acid (Ácido oleanólico)

PPARγ	Peroxisome-proliferator activator receptor-gamma (receptor gamma del activador de proliferación del peroxisoma)
Ppm	Partes por millón
PUFA	Polyunsaturated fatty acids (ácidos grasos poliinsaturados)
Rho GTPasas	Hidrolasa rho del guanosín trisfosfato
SFA	Satured fatty acids (ácidos grasos saturados)
SOD	Superóxido dismutasa
TAMs	Tumor-associated macrophages (macrófagos asociados a tumores)
Th1/Th2	T helper cell 1 or 2 (células T cooperadores tipo 1 o 2)
TIMPs	Tissue inhibitors of metalloproteinases (inhibidores de las metaloprotesas propios del tejido)
TNF-α	Tumor necrosis factor –α (factor de necrosis tumoral alpha)
UV	Uvaol
VEGF	Vascular endothelial growth factor (factor de crecimiento endotelial vascular)
ΔΨm	Potencial de membrana mitocondrial
μM	Micromolar

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INTRODUCCIÓN

Introducción

1. Cáncer de mama

1.1 Epidemiología del cáncer de mama

El cáncer fue, según el último informe de la Organización Mundial de la Salud (OMS), una de las principales causas de muerte en los países desarrollados en el período comprendido entre 2000 y 2011. En 2012 se contabilizaron un total de 14,1 millones de nuevos casos, unos 8,2 millones de muertes por cáncer y 32,6 millones de personas viviendo con la enfermedad (GLOBOCAN 2012).

Entre los tipos de cáncer que más muertes provocan cada año se encuentran los de pulmón, hígado, estómago, colon y mama.

El cáncer de mama es el segundo más común en términos de incidencia mundial, y el primero en mujeres, con 1,67 millones de nuevos casos diagnosticados en 2012 (25% del total de canceres diagnosticados). Este tipo de cáncer es el más común en mujeres tanto de regiones desarrolladas como de las menos desarrolladas, siendo la incidencia mucho más acusada en las regiones desarrolladas. Pero en términos de mortalidad, el cáncer de mama es el más mortífero entre todos los cánceres en las regiones menos desarrolladas (324.000 muertes, 14,3% del total) mientras que es la segunda causa de muerte por cáncer en las regiones desarrolladas (198.000 muertes, 15,4% del total), por detrás del cáncer de pulmón (figura 1).

Entre los factores de riesgo descritos hasta ahora para padecer un cáncer de mama se encuentran: la edad del individuo, antecedentes familiares, edad de aparición de la menarquia, edad de aparición de la menopausia, edad en la que se tuvo el primer hijo, obesidad, nuliparidad, lactancia materna, estilo de vida (consumo de tabaco y alcohol) (Rockhill 2001; Horn *et al.* 2014) y la dieta (Sieri *et al.* 2004; Fung *et al.* 2005); estando asociados algunos de ellos con el origen molecular de los distintos subtipos de cáncer de mama estudiados (Horn *et al.* 2014).

1.2 Carcinogénesis

Se denomina carcinogénesis u oncogénesis a la transformación de una célula normal en una célula cancerosa. Aunque en determinados tipos de cáncer, cambios en el estroma pueden preceder al desarrollo de una carcinogénesis sin la presencia de células cancerosas (Bhowmick *et al.* 2004; Ariztia *et al.* 2006), en el caso de tumores de origen epitelial, como el de mama, el origen del tumor es la transformación de

células epiteliales en células neoplásicas mediante la adquisición de varias características o mutaciones procancerígenas, que reprograman la célula hasta un proceso de división incontrolada (Siemann 2010). Más adelante veremos que estas mutaciones pueden ser debidas a la presencia de agentes procancerígenos, como por ejemplo las especies reactivas de oxígeno (EROs).
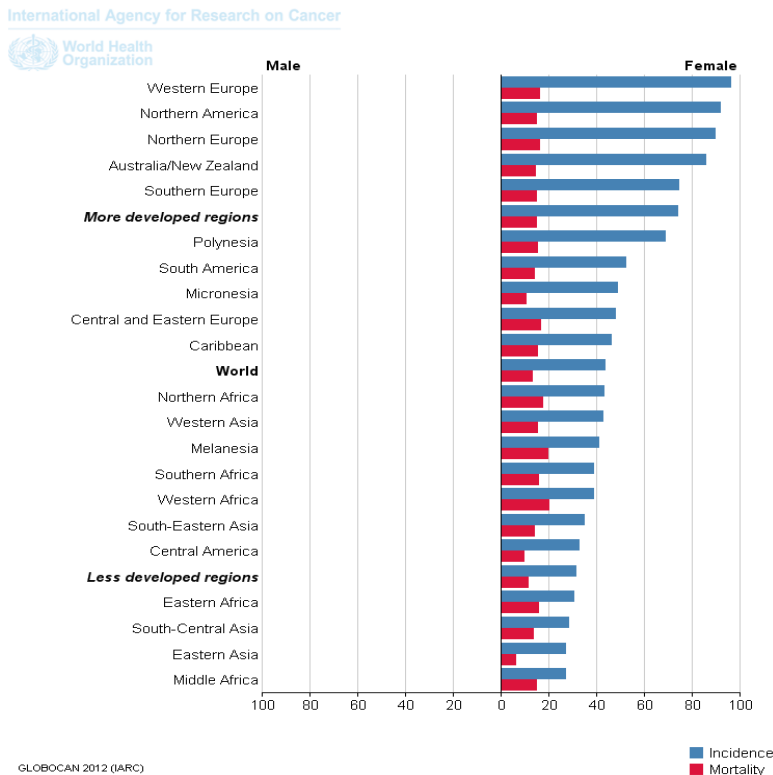
Figura 1. Incidencia y mortalidad del cáncer de mama por regiones. Ratio estándar expresado por 100.000 (GLOBOCAN 2012).

A la división incontrolada de una célula se le conoce con el nombre de hiperplasia o hiperproliferación celular. Este tipo de células presentan patrones de baja o nula regulación del crecimiento. Lesiones génicas las predisponen a tener ventaja en el crecimiento y división celular sobre las células normales del mismo tejido. Por tanto, un elemento importante en la trasformación maligna de la célula somática es la pérdida de los mecanismos reguladores de control, que tiene su crecimiento controlado y no se divide hasta que se señaliza lo contrario. Alteraciones genómicas,

proteómicas, post-transduccionales y epigenéticas son las responsables de la activación oncogénica y la inhibición de genes supresores de tumores.

Las células cancerosas no sólo pierden las pautas de crecimiento y división celular, así como los puntos de control del ciclo celular, sino que también pierden la regulación de la inhibición del crecimiento por contacto. De esta forma, las células cancerígenas siguen creciendo descontroladamente inclusive a través de las membranas basales que rodean otros órganos, invadiendo los espacios extracelulares (matriz extracelular) que rodean a los tejidos y órganos (Siemann 2010).

1.2.1. Hipoxia y angiogénesis

El remodelado de la matriz extracelular y los cambios en las moléculas de adhesión de la célula cancerígena promueven su motilidad, de manera que a medida que las células cancerígenas van dividiéndose, adquieren mayor motilidad para poder invadir los espacios anexos a ellas. Esto lo consiguen con cambios tanto de reordenamiento de la actina del citoesqueleto, lo que favorece la plasticidad y motilidad, como con la inhibición o baja expresión de proteínas de adhesión.

La adquisición de todas estas características da lugar a la proliferación de células cancerígenas que van avanzando y creciendo progresivamente en la matriz extracelular, lugar al que los vasos sanguíneos del tejido normal no pueden acceder para oxigenar y nutrir a las células cancerosas.

Como consecuencia, las células cancerosas más distales a la zona donde se formó el tumor crecen con deficiencia de oxígeno (regiones hipóxicas). Estas células resuelven el problema convirtiendo o cambiando la glucólisis aeróbica a glucólisis anaeróbica, de forma que obtienen la energía que necesitan para proliferar y además esquivan los efectos citotóxicos de la privación de oxígeno (Siemann 2010). De hecho, los productos generados en la glucólisis anaeróbica contribuyen a la degradación de la matriz extracelular, facilitan la motilidad celular e incrementan el potencial metastásico.

Esta adaptación metabólica ofrece una ventaja adicional de la proliferación de la célula cancerosa: la supresión apoptótica. La glucólisis anaeróbica y la supresión apoptótica parecen estar relacionadas mediante la mitocondria. De hecho, algunas enzimas glucolíticas regulan también procesos apoptóticos y algunas oncoproteínas inducen la expresión de enzimas glucolíticas. A causa de esta glucólisis, en la mitocondria se generan EROs junto con un flujo de salida de H^+, lo que causa un potencial de membrana ($\Delta\Psi m$) negativo. Estos cambios mitocondriales causan efectos posteriores en la producción de energía, ya que la mitocondria regula funciones críticas como el control de la concentración de Ca^{2+}, así como el equilibrio

de oxidación-reducción de las EROs; ambas funciones relacionadas con la apoptosis celular y quizás con la inhibición de la apoptosis en el cáncer.

Las EROs influyen directamente en la polarización de la membrana de la mitocondria cambiando el voltaje de los canales de potasio (K), permitiendo así que la mitocondria se hiperpolarice y disminuyendo con el tiempo la eficacia de los canales de K.

Una de las diferencias entre las células normales y las células del cáncer es precisamente ésta, la diferencia de polarización entre sus mitocondrias, por lo que algún agente que modifique este estado hasta su situación original (el potencial de membrana de la célula no cancerígena) podría influir en la reactivación de la apoptosis y la inhibición del crecimiento; siendo por tanto un magnífico agente quimioterápico (Bonnet *et al.* 2007).

Una vez que la célula ha modificado su metabolismo para poder escapar a la apoptosis y poder metabolizar energía sin oxígeno, necesita una fuente que la provea de nutrientes para seguir proliferando. Los vasos sanguíneos del tejido normal no pueden proveer de nutrientes a las células más distales del tumor. Como resultado de la glucólisis anaeróbica los subproductos del ácido láctico se secretan a la matriz, dando paso a una acidificación generalizada. Esta acidificación contribuye a una retención de líquidos y al consiguiente incremento de la presión intersticial. Debido al crecimiento descontrolado de estas células se necesita algún mecanismo que aporte oxígeno (no todas han llevado a cabo el cambio metabólico de glucólisis aeróbica a anaeróbica) y nutrientes. Aquí las células adquieren otra ventaja fenotípica importante, la habilidad para inducir angiogénesis.

Las células cancerosas sobreexpresan y liberan a la matriz factores proangiogénicos que desestabilizan a las células endoteliales del tejido e inducen el crecimiento de nuevos vasos sanguíneos. Este proceso de formación de nuevos vasos sanguíneos se denomina angiogénesis. Las células endoteliales proliferan alrededor de la zona donde se han segregado estos factores angiogénicos quimioatrayentes para formar una nueva red capilar que "alimentará" a la masa del tumor. Pero a diferencia del tejido normal vascularizado, la red capilar que se forma alrededor del tumor no está organizada y tiene poca integridad vascular.

Uno de los factores proangiogénicos que se cree que es el mayor inductor de angiogénesis tumoral es el factor de crecimiento endotelial vascular o VEGF (vascular endothelial growth factor). La expresión de VEGF está implicada en distintos tipos de cáncer, correlacionándose con la angiogénesis, la agresividad tumoral y una mala evolución de los pacientes. Además, VEGF es predictor de metástasis.

De hecho, VEGF es importante en microambientes acidificados, dónde se induce el lisado de vesículas, de esta forma se liberaría al medio una vez sintetizada dentro de la célula cancerosa, contribuyendo a un mecanismo de retroalimentación: el tumor induce la hipoxia, ésta da lugar a la acidificación celular y ésta última al lisado de vesículas, algunas de ellas con VEGF, lo que activa la vascularización del tumor (Siemann 2010)(figura 2).

Figura 2. Esquema de retroalimentación de los procesos por los cuáles el tumor consigue la formación de nuevos vasos sanguíneos.

Los nuevos tratamientos antiangiogénicos utilizados en cáncer se centran precisamente en el VEGF, administrando a los pacientes agentes que bloquean la señalización de vascularización (inhibidores de VEGF). Estas terapias han mejorado la evolución de los pacientes con cáncer, pero plantean nuevas complicaciones como la hipertensión. Los inhibidores de VEGF promueven la disfunción endotelial, así como un incremento en la resistencia vascular, lo cual viene generado por una señalización deficiente del óxido nítrico (NO), una reducción de la producción de prostaciclina, una sobreproducción de la endotelina-1, estrés oxidativo y rarefacción. Debido a ello, en

algunos casos incluso se debe de interrumpir el tratamiento por el efecto hipertensivo que producen (Small *et al.* 2014).

Generalmente se cree que un tumor no puede crecer más de unos pocos milímetros cúbicos sin inducir la generación de nuevos vasos sanguíneos. Una vez que el tumor lo consigue, se asegura un aporte de nutrientes mucho más constante, y puede así crecer más efectiva y rápidamente. El tumor va necesitando espacio adicional y por tanto necesita invadir los tejidos adyacentes. Para ello las células que conforman el tumor empiezan a intentar digerir la matriz extracelular adyacente y forzar a la reorganización del tejido epitelial normal y de los elementos estromales.

1.3 *La matriz extracelular*

El estroma está separado del tejido epitelial y del endotelio por una membrana basal, un tipo especializado de matriz extracelular conformada por colágenos de tipo IV, laminina y proteoglicanos en su mayoría.

La matriz extracelular se compone de varios tipos celulares y proteínas que ayudan a mantener la organización de los órganos y tejidos. De hecho está formada en su mayoría por una mezcla de colágeno, fibronectina, laminina, hialuronano, plasminógeno, proteasas, etc. que conjuntamente forman una estructura inflexible que será atacada por las células del tumor. Entre las células que podemos encontrar en la matriz se encuentran los fibroblastos (responsables del remodelado de la matriz), células endoteliales, células hematopoyéticas y células inmunitarias (quienes supervisan y monitorizan la presencia de agentes externos a la matriz).

En esta matriz extracelular también encontramos citoquinas y proteínas remodeladoras de la matriz extracelular secretadas por las células del tumor. La matriz se va degradando gradualmente por la acción de estas citoquinas y proteínas, que a su vez se activan debido a la interacción con otras proteasas y enzimas activadoras presentes en el microambiente de la matriz. Dentro de las proteínas que remodelan la matriz encontramos un conjunto de metaloproteasas de la matriz ancladas a la membrana (MMPs, membrane-anchored matrix metalloproteinases) responsables de la degradación de los componentes de la matriz. Junto con las MMPs actúan factores como endoglucosidasas y serinproteasas (activadores de plasminógeno, uroquinasas, trombinas o plasminas). Interesante es el hecho de que algunos de los factores mencionados no son secretados por las células tumorales, sino por las células normales del tejido. De hecho las MMP-9, MMP-12 y la MMP-8 son liberadas por células inflamatorias infiltradas en la masa tumoral, junto con la

interleuquina 1 beta (IL-1β) y el factor de necrosis tumoral alpha (TFN-α; tumor necrosis factor-α) que estimulan a los fibroblastos para que produzcan más MMPs. Además de estos fibroblastos y células inmunitarias, otras células estromales se encargan de secretar al medio más MMPs durante la carcinogénesis.

Las funciones de estas MMPs son diversas, pero en conjunto degradan la matriz extracelular y a otras proteínas del microambiente tumoral (factores de crecimiento, citoquinas y receptores) con el fin de establecer un microambiente adecuado que rodee a las células tumorales. La MMP-7, por ejemplo, expresada por las células tumorales de mama, se encarga de degradar la matriz extracelular, rompe la membrana basal y se une a la E-cadherina de las células normales, para debilitar las conexiones entre las células epiteliales del tejido mamario. Las MMPs están presentes en el microambiente en forma de zimógeno, y es necesaria su activación mediante otras MMPs o moléculas relacionadas. Las MMPs por sí solas no son capaces de llevar a cabo la degradación de la matriz, de hecho son reguladas por los inhibidores de metaloproteasas (TIMPs; tissue inhibitors of metalloproteinases) pero junto con otra clase de señales y moléculas presentes en el microambiente del tumor, pueden derivar en el crecimiento y el establecimiento del tumor mamario.

Los fibroblastos estromales asociados al cáncer juegan un papel fundamental en la transformación, proliferación e invasión del cáncer (tres de los puntos clave en el cáncer). Estos fibroblastos segregan factores de crecimiento y quimiocinas que producen cambios críticos en la remodelación de la matriz extracelular y contribuyen a un mayor incremento de la proliferación e invasión, creando así el ambiente físico del tumor en crecimiento. Entre las moléculas liberadas por los fibroblastos a la matriz se encuentra la proteína 1 secretada por fibroblastos (FSP1, fibroblast secreted protein-1) que parece estar relacionada directamente con el crecimiento del tumor y con la metástasis del mismo.

Pero la degradación de la matriz no sólo la llevan a cabo fibroblastos guiados por la señalización de las células tumorales, sino que son las propias células tumorales las que segregan moléculas relacionadas con la degradación de la matriz, como las MMPs.

El balance de MMPs, proteínas, citoquinas, enzimas degradativas y demás moléculas secretadas tanto por el tumor como por las células normales adyacentes, da lugar a un remodelado de la matriz extracelular. Diferentes tipos celulares (epiteliales, estromales, inmunes) contribuyen a la síntesis de colágeno formando el estroma peritumoral que está caracterizado por estar desorganizado, cómo todo lo que rodea al desarrollo del tumor (Siemann 2010).

1.4 La inflamación en el proceso tumoral

Fue Rudolf Virchow, en 1863, el primero en descubrir la presencia de leucocitos en tejidos neoplásicos y en asumir que el origen de un tumor se producía en las zonas donde una inflamación crónica previa tenía lugar. Actualmente parece obvio que un tumor no es capaz de desarrollarse únicamente por la proliferación de células malignas, sino que deben darse una serie de condiciones que permitan el desarrollo tumoral, entre ellas un proceso de inflamación (Coussens y Werb 2002).

Ya hemos visto que en el microambiente del tumor se encuentran determinados tipos de células que interactúan entre sí y con el tumor para producir señales y moléculas que ayudarán al desarrollo y establecimiento del tumor. Entre ellas se encuentran células del sistema inmune, que secretan factores de crecimiento, citoquinas y quimiocinas que estimulan la proliferación celular y generan EROs que dañan el ácido desoxirribonucleico (ADN) de las células y promueven la progresión del tumor. También se encargan de secretar enzimas proteolíticas que ayudan al remodelado de la matriz celular y la angiogénesis.

Una de las primeras células en responder a las señales de crecimiento secretadas por el tumor son las células mastocíticas o mastocitos, que responden liberando VEGF, serin proteasas y MMP-9 (Siemann 2010).

Pero las células que en mayor número aparecen en el seno del tumor son los macrófagos. Estas células proceden de monocitos inactivados presentes en sangre. Podemos encontrarlos tanto en el microambiente que rodea al tumor como dentro de la masa tumoral y se denominan TAMs (tumor-associated macrophages; macrófagos asociados a tumores).

Estudios recientes apuntan a que los mecanismos básicos en la progresión del tumor de mama son similares a los del proceso de reorganización del desarrollo de la glándula mamaria; de hecho se considera que un tumor sólido posee una estructura de "órgano" en la que coexisten tanto las células tumorales como las normales, intercambiando señales para el desarrollo del tumor. Y en toda esta formación se encuentran macrófagos, que son los grandes contribuyentes a la progresión del tumor. De hecho, pueden incluso incrementar la supervivencia y la capacidad de proliferación de las células tumorales, promoviendo su motilidad, la capacidad de intravasación e invasión, conduciendo la angiogénesis y mediando en la inmunosupresión y la reorganización de la matriz extracelular (Laoui *et al.* 2011).

1.4.1. Relevancia clínica de los TAMs en cáncer de mama

Varios estudios, sobre todo en grupos de carcinomas ductales invasivos de mama, revelan que existe una correlación positiva entre altos niveles de macrófagos infiltrados en la masa tumoral y la expresión de quimioatrayentes para monocitos/macrófagos por parte de las células tumorales. Una alta densidad de TAMs está asociada a una alta densidad vascular, sugiriendo que los TAMs poseen una actividad proangiogénica en tumores humanos; de hecho la liberación de VEGF está relacionada positivamente con los niveles de TAMs (Siemann 2010).

En cáncer de mama, éstos parecen estar relacionados con una mala prognosis y una baja supervivencia (Heusinkveld y Van der Burg 2011; Zhang *et al.* 2012). Unos índices altos de macrófagos infiltrados parecen estar relacionados directamente con un alto grado de desarrollo del tumor (estadíos III y IV), un estatus de baja expresión de receptores de progesterona y estrógenos y una alta actividad mitótica del tumor (Laoui *et al.* 2011).

1.4.2. Estados de activación de los TAMs

Los macrófagos son células con una importante plasticidad. Normalmente están implicados en procesos del desarrollo tisular y su homeostasis, así como la curación de heridas en los tejidos, la inflamación y la inmunidad.

Para poder actuar en tan diversos procesos, los macrófagos adoptan estados de activación o polarización dependientes de los estímulos que éstos reciban del microambiente. Actualmente se consideran no solo dos únicos estados de activación, sino un rango de activación entre los estados descritos, dependiendo del tipo de señalización del microambiente (Biswas y Mantovani 2010). Los dos estados de activación a los que hago referencia son (Mantovani y Sica 2010; Laoui *et al.* 2011):

- <u>M1 o clásicamente activados</u>: macrófagos activados mediante señales producidas por las interleuquinas (IL) o citoquinas del tipo Th1 (T helper 1 cells; células T cooperadoras tipo 1), como interferón gamma (INF-γ) y TNF-α, y/o por el reconocimiento de patrones moleculares asociados a patógenos o señales endógenas de peligro. Estos macrófagos tienen actividad tumoricida y provocan reacciones de destrucción de tejidos, desarrollando una respuesta de tipo Th1 o citotóxica (tabla 1).
- <u>M2 o alternativamente activados</u>: macrófagos activados mediante señales producidas por IL del tipo Th2 (T helper 2 cells; células T cooperadoras tipo 2), como IL-4 e IL-13. Estos macrófagos regulan las señales de tipo Th2 para contribuir a la cicatrización de la herida y al control de la inflamación. Se caracterizan por su baja expresión del complejo mayor de

histocompatibilidad II (MHC II; major histocompatibility complex II) y una muy reducida actividad antimicrobiana y tumoricida. Por otra parte, promueven la angiogénesis, la sobreproducción de IL-10 (citoquina antiinflamatoria), el remodelado del tejido y la progresión tumoral (tabla 1).

En la mayoría de tumores estudiados, los TAMs son mayoritariamente macrófagos del tipo M2 (Mantovani y Sica 2010), de manera que incluso en las zonas de hipoxia dentro del tumor, los macrófagos existentes poseen un fenotipo M2 (Van Overmeire *et al.* 2014), relacionándolos directamente con zonas donde el tumor está creciendo y desarrollándose. En algunos tipos de cáncer (como pulmón y cérvix) un alto índice de macrófagos infiltrados se relaciona con un mejor pronóstico. Concretamente, un alto índice en el ratio M1/M2, es decir macrófagos polarizados o activados en su mayoría a M1 (Heusinkveld y Van der Burg 2011).

Pero hay que tener en cuenta que la mayoría de los estudios que relacionan los distintos tipos de fenotipos encontrados en los macrófagos infiltrados en el tumor, están realizados en tumores con estadio de progresión altos, mientras que no hay suficientes estudios realizados en tumores de relativa nueva formación.

ESTADO DE ACTIVACIÓN	MARCADORES	MOLÉCULAS SECRETADAS	ACCIÓN QUE EJERCEN
M1	$CD68^+$ $CD14^+$ MHC II^{alto} $CD163^{bajo}$ $CD206^{bajo}$	EROs NO (por iNOS) IL-12p70 IRF-5	Activación respuesta Th1
M2	$CD68^+$ $CD14^+$ MHC II^{bajo} $CD163^{alto}$ $CD206^{alto}$	MMP-1 MMP-2 MMP-9 IL-10	Activación respuesta Th2

Tabla 1. Resumen de las características más relevantes de los dos estados de activación de los macrófagos y la acción que ejercen en el tumor (Heusinkveld y Van der Burg 2011).

Además, los TAMs son un reflejo del microambiente del tumor, donde también podemos encontrar distintos tipos celulares del sistema inmune: cómo células T reguladoras que ejercen un impacto negativo en la supervivencia del paciente, o células T citotóxicas que promueven la regresión del tumor y la supervivencia del paciente. Los macrófagos no sólo contribuyen a la inflamación local, también son capaces de activar una respuesta de las células T efectiva en los nódulos linfáticos para la inducción de apoptosis en el tumor (Heusinkveld y Van der Burg 2011).

Como anteriormente se ha descrito, el estado de activación del macrófago puede inducirse dependiendo de las señales del microambiente y de las moléculas que secreten las células localizadas dentro o fuera del tumor. Pero este estado de activación no es permanente, es decir, en la vida útil del macrófago puede darse más de un estado de activación, de manera que la producción de un determinado tipo de moléculas puede reactivar un macrófago polarizado como M1 a un estado de polarización M2 y viceversa. De hecho los macrófagos M1 expresan IRF-5 (factor de regulación del interferón 5; interferon regulatory factor 5), molécula responsable de la producción de interferón tipo 1 y la represión de IL-10; cuya expresión forzada en un macrófago M2 obliga al macrófago a la repolarización a M1.

1.4.3 Papel de los macrófagos en el proceso tumoral

En el desarrollo del tumor se necesita un aporte de nutrientes y un mecanismo de descarga de desechos metabólicos y de dióxido de carbono. Estas necesidades se ven cubiertas mediante la vascularización del tumor. Los macrófagos son la perfecta ayuda en este proceso, especialmente los activados a M2, pues son ellos los que se encargan de secretar moléculas para la remodelación del tejido y además producen VEGF. Se sabe que el tumor atrae a monocitos de la sangre mediante la secreción de quimioatrayentes (de los tipos CXC, CC, C y CX3C) que ejercen sus efectos en células diana mediante miembros de la familia de las 7-transmembrana y receptores acoplados a proteínas G. Mediante la secreción de estos quimioatrayentes o quimiocinas el tumor regula el reclutamiento de leucocitos, manipula la respuesta tumoral, regula la angiogénesis, el crecimiento tumoral y controla el movimiento de las células tumorales en la metástasis (figura 3). Estas quimiocinas atraen al macrófago hacia el tumor, una vez allí el propio macrófago pasa a secretar quimiocinas, MMPs que atraen a más monocitos de la sangre e incluso VEGF, que aparte de dirigir la neovascularización del tumor, parece tener un papel como quimioatrayente para monocitos en el tumor de mama y dirigir los movimientos de los TAMs dentro del tumor.

Estos monocitos se agrupan en las regiones hipóxicas del tumor, debido a un gradiente quimioatrayente producido por las células tumorales en hipoxia. En estas

regiones encontramos también un alto índice de VEGF expresado por las células tumorales y los macrófagos. De hecho, la presencia de áreas del tumor ricas en VEGF en tumor de mama se relaciona con regiones poco vascularizadas y con presencia de gran número de TAMs.

Aunque los mecanismos por los cuales los macrófagos permanecen retenidos en las regiones de hipoxia no están del todo claros aún, parece que existen varios procesos relacionados o responsables con este hecho. Un mecanismo inicial dónde la hipoxia y/o TNF-α inactiva la ruta de las MAPK (mitogen-activated protein kinase; quinasa proteica activada por mitógeno), evitando así la señalización para la migración de TAMs; y una segunda vía donde TNF-α e INF-γ modulan la expresión de quimioatrayentes para evitar la migración de los TAMs (Murdoch *et al.* 2004).
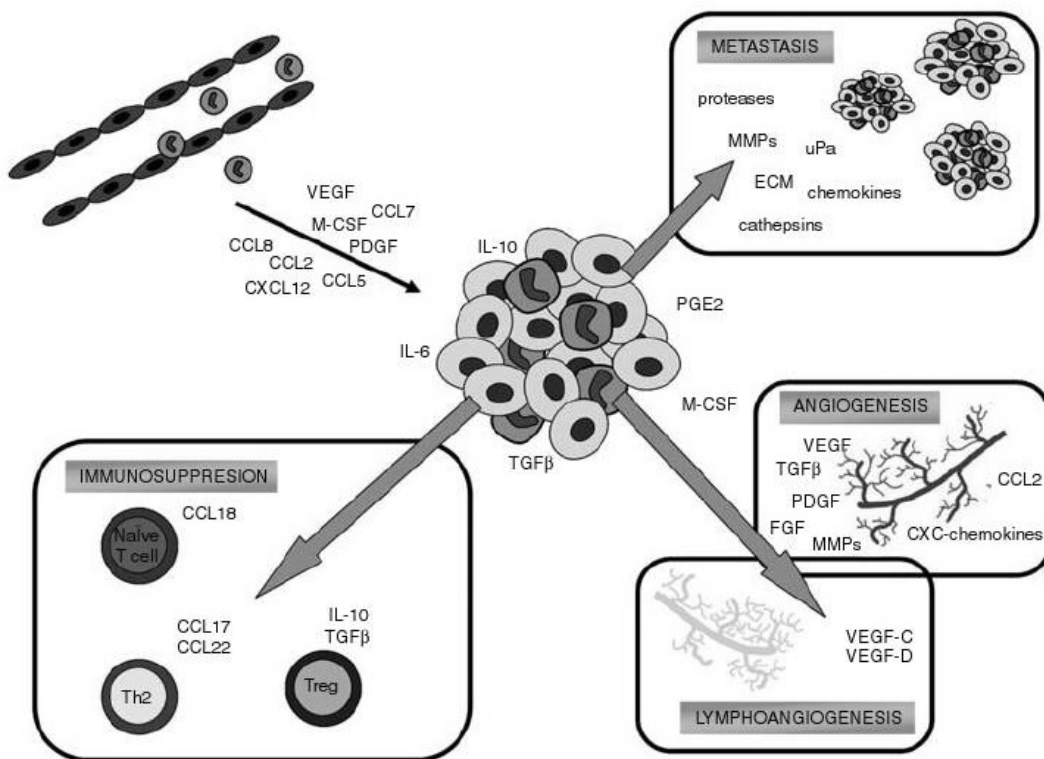
Figura 3. Mecanismos de reclutamiento de los TAMs y sus funciones protumorales. Quimiocinas (CCL2, CCL5, CCL7, CCL8, CXCL12) y factores de crecimiento (M-CSF, PDGF y VEGF) activan el reclutamiento de monocitos sanguíneos en el sitio del tumor, dónde se diferencian a TAMs. Las señales del microambiente tumoral, como IL-10, PGE2, M-CSF, TGFβ e IL-6 promueven la polarización de los TAMs al fenotipo M2. Los TAMs promueven el crecimiento tumoral y su progresión mediante diferentes mecanismos. Los TAMs inhiben las respuestas antitumorales con la secreción de citoquinas

inmunosupresoras, como la IL-10 y el TGFβ, y el reclutamiento de células T no activas, mediante la citoquina CCL18. Los linfocitos Th2 (T helper 2; T cooperadores 2) y Treg (T reguladores) mediante las citoquinas CCL17 y CCL22 también son atraídos al lugar. Los TAMs también contribuyen tanto a la formación de vasos sanguíneos como linfáticos mediante la liberación de factores angiogénicos (VEGF, FGF, TGFβ, PDGF, MMPs y quimiocinas) y factores linfoangiogénicos (VEGF-C, VEGF-D). Más adelantes estos mismos TAMs promueven la invasión del tumor y la formación de metástasis mediante la secreción de proteínas de la matriz, enzimas remodeladoras de la matriz (MMPs, proteasas, etc) y sus activadores (quimiocinas) (Siemann 2010).

Una vez que los macrófagos son reclutados en las áreas de interés por el tumor, todo el conjunto de células que conforman el entramado tumoral, como macrófagos, leucocitos atraídos por los macrófagos, otras células inmunes, fibroblastos, células endoteliales y las propias células tumorales interactúan entre sí para el propio crecimiento y desarrollo del tumor. Los TAMs son capaces de ayudar en el crecimiento, pero también en la angiogénesis, linfogénesis (formación de nuevos vasos linfáticos), migración e invasión de las células tumorales.

1.5 Metástasis

Aproximadamente un 90% de las muertes totales atribuidas al cáncer son a causa de la metástasis, constatando que se necesitan terapias más efectivas contra el cáncer y su propagación a otros órganos (Siemann 2010).

La metástasis representa el estadio final y más devastador de la enfermedad. La muerte se produce por el fallo de los órganos colonizados, síndromes paraneoplásicos y/o los efectos secundarios de la terapia utilizada. Estudios genéticos realizados en el tumor primario y en sus metástasis han puesto de manifiesto la modulación de cascadas bioquímicas en la propagación del tumor diseminado, cuya activación o inhibición se realiza dependiendo de los diferentes estados de la diseminación tumoral. Generalmente se ha pensado que la metástasis se originaba una vez que el tumor seleccionaba aquella clona de células que podía diseminarse en la distancia y colonizar otro órgano distante. Pero nuevos estudios apuntan a que dependiendo del tumor y diferentes características de éste, es al principio de la carcinogénesis cuando determinadas células abandonan la lesión primaria del tumor y salen a colonizar un órgano diana en el que, paralelamente al tumor primario, desarrollan una metástasis (Pani *et al.* 2010). Estas células estarían programadas (mediante la expresión de determinados genes) dependiendo de factores como el origen celular, propiedades intrínsecas del tumor primario y afinidades de tejido para colonizar no sólo órganos específicos (órganos diana), sino para determinar el curso temporal y la severidad de la metástasis en órganos vitales (Chiang y Massague 2008; Nguyen *et al.* 2009).

Parece que sólo una minoría de las células malignas desarrolla características metastásicas, y de éstas una fracción aún más pequeña tiene éxito. Pero las razones por las cuáles estas células abandonan el tumor primario no están claras aún. Se desconoce la presión ambiental que determina que desarrollen características metastásicas, aunque se conoce que el ambiente tumoral puede serles hostil, debido al poco aporte de nutrientes y oxígeno que pueden recibir, así como la inflamación y los ataques inmunológicos. De hecho se sabe que la inflamación y la hipoxia promueven metástasis (Bertout *et al.* 2008; Mantovani 2009), razones por las cuales células del tumor primario podrían convertirse en metastásicas como vía de escape.

La célula metastásica debe adoptar mecanismos que le permitan el escape del tumor primario y después recorrer una distancia que es importante desde el punto de vista celular. La secuencia típica que debe adoptar es:

1. La pérdida de la polaridad celular y la separación de las células circundantes del tejido primario.
2. El incremento de su interacción con la matriz extracelular mediante integrinas.
3. La migración mediante la matriz extracelular hasta vasos linfáticos y sanguíneos.

Este proceso se denomina transición epitelio mesénquima (EMT; epithelial to mesenchymal transition) y ha sido investigado profundamente como primer proceso en la metástasis. Este proceso también se produce durante el desarrollo embrionario, en el que las células migran a diferentes localizaciones dependiendo de gradientes morfogenéticos.

En la EMT se pierde la adhesión célula-célula mediante la baja producción de E-cadherina y aparece una capacidad creciente de interacción con las proteínas de la matriz, degradando las integrinas mediante MMPs, y moviéndose mediante una reordenación de su propio citoesqueleto coordinado con proteínas de la familia de las Rho GTPasas.

Otra estrategia celular para la migración celular es la invasión ameboidea, donde las células pierden su polaridad y se despegan de la matriz extracelular usando la contracción de la actina cortical para su propulsión, mecanismo de menor gasto energético que el anterior.

El siguiente paso crítico es la supervivencia de la célula metastásica en el torrente sanguíneo. La mayoría de las células normales entran en apoptosis cuando se despegan de las matrices del tejido, pero las células metastásicas deben de

desarrollar mecanismos moleculares que evadan estas señales, para no solo sobrevivir sino proliferar antes de llegar al sitio metastásico.

Una vez la célula ha llegado a su destino se produce una extravasación desde los vasos al tejido. En este momento la célula se adapta y atraviesa la matriz extracelular llegando al tejido, dónde creará su nicho para desarrollar la metástasis.

Esta célula debe poseer capacidad para proliferar, estimular angiogénesis e intercambiar componentes con el nuevo microambiente, incluyendo células parenquimales, estromales e inflamatorias. Del balance entre apoptosis, proliferación celular, pérdida de la neoangiogénesis y entrada de la célula en estado quiescente, se desarrolla el fenómeno de dormancia celular, en el cuál la célula no prolifera; estado que el tumor aprovecha para diseminar células tumorales durmientes con el fin de desarrollar tumores secundarios en otros órganos.

La ganancia de potencial metastásico en una célula está relacionado directamente con una reprogramación genética celular que desemboca en el desarrollo de las metástasis (Pani *et al.* 2010).

1.6   *Estrés oxidativo en cáncer*

Se define estrés oxidativo como el desequilibrio producido en la célula debido a una alta producción de EROs y una baja eficiencia del sistema celular para desintoxicar o reparar el daño resultante. Este equilibrio es dependiente 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 4).

La mitocondria es la principal fuente de producción de EROs debido a la reducción del oxígeno por un electrón, una reacción química de la cadena de respiración de la mitocondria. 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.
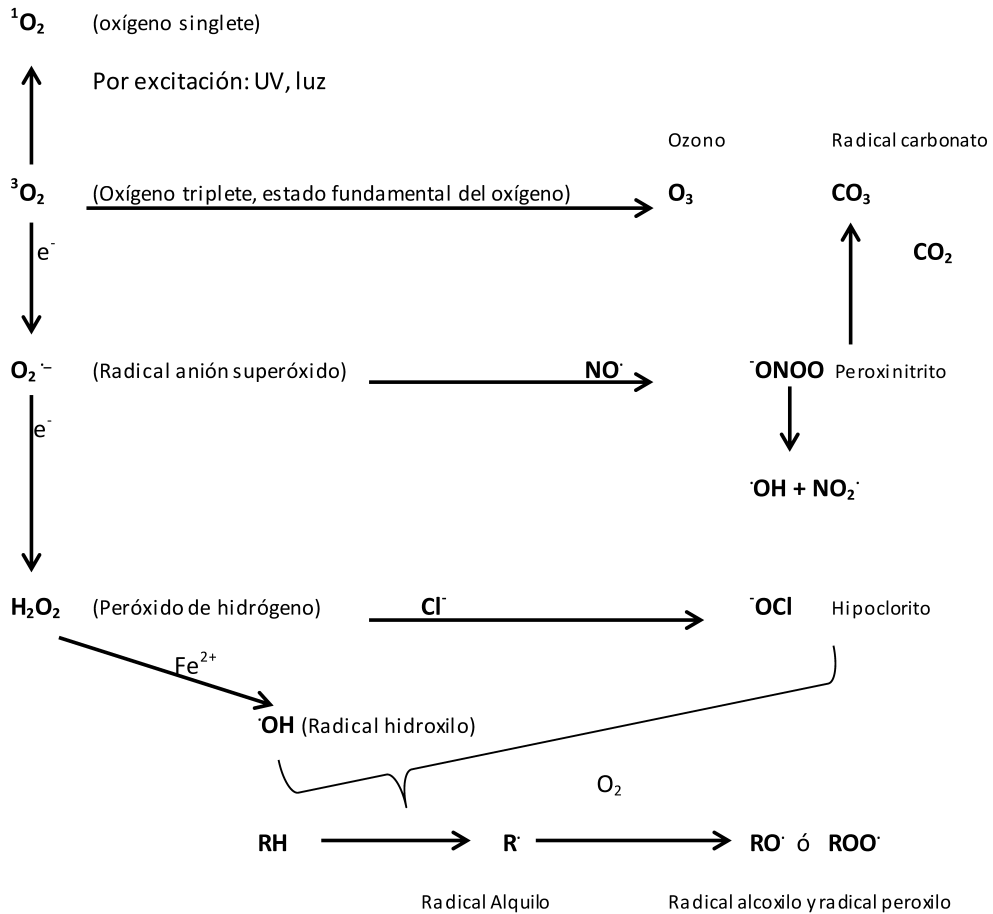
Figura 4. Principales derivados de EROs y componentes orgánicos con los que reaccionan (RH, molécula orgánica) (Bartosz 2009).

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 endógenas (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 relación al ADN. 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.

Aparte de su interacción con el ADN, estas especies reactivas pueden también oxidar a proteínas y a los lípidos celulares. Estas interacciones normalmente producen la pérdida de la función proteica. 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 las enzimas celulares antioxidantes y con antioxidantes que pueden reaccionar rompiendo estos radicales, como por ejemplo la vitamina C, el β-caroteno, ubiquinonas, etc (Lopaczynski y Zeisel 2001).

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, ya que no poseen mecanismos antioxidantes. 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 (Bonnet *et al.* 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 utilizados.

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 *et al.* (2000) observaron que una inhibición selectiva de la SOD (superóxido dismutasa) acababa con las células tumorales, pero no con 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 eran 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.

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.

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 (como antes se ha descrito). El no poseer estas 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 (activating-protein-1; proteína de activación 1), NF-κβ, p53 e HIF (hipoxia-induced factor; 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 *et al.* 2010).

2. Dieta Mediterránea

Son numerosos los estudios que revelan los beneficios de la dieta mediterránea. En 1989 se describió el modelo alimentario de la población española, apuntando que los patrones tradicionales de alimentación debían de perpetuarse en el tiempo por los beneficios saludables de éstos (Moreiras-Varela 1989).

En 2000, Trichopoulou *et al*. sugieren que ante enfermedades como el cáncer, tanto la dieta mediterránea como las tradiciones de los países mediterráneos repercutían en su incidencia (como por ejemplo el de mama, endometrio o próstata). De hecho se observó que en países mediterráneos la probabilidad de padecer cualquier tipo de cáncer era menor que en los países escandinavos, el Reino Unido y los Estados Unidos (Trichopoulou *et al.* 2000).

Actualmente podemos encontrar estudios dirigidos a demostrar los beneficios de esta dieta para prevenir cierto tipo de enfermedades como el PREDIMED (Prevención

con Dieta Mediterránea). Este estudio detalla los efectos beneficiosos del consumo de una dieta Mediterránea en la prevención de enfermedades cardiacas (Estruch *et al.* 2013; Martínez-González *et al.* 2014; Ros *et al.* 2014; Whayne 2014), así como en diabetes tipo 2 (Lasa *et al.* 2014), en la enfermedad del síndrome metabólico (Mayneris-Perxachs *et al.* 2014), aumenta la longevidad (Vasto *et al.* 2013) o incluso previene la depresión (Sánchez-Villegas *et al.* 2013).

La dieta mediterránea está compuesta principalmente por alimentos vegetales (frutas, verduras, legumbres, champiñones y frutos secos), cereales (pasta, arroz y productos integrales), alimentos frescos y de temporada, lácteos (yogur y queso principalmente), pescado en abundancia, y huevos y vino con moderación. Como eje central y protagonista se encuentra el aceite de oliva, principal grasa de adición (figura 5).

Figura 5. Pirámide de la dieta mediterránea (Fundación Dieta Mediterránea 2010).

Esta dieta mediterránea se complementa con un estilo de vida saludable, en el cuál la actividad física debe de ser diaria.

El aceite de oliva es la principal grasa utilizada en esta dieta, siendo también origen de un gran número de aportaciones saludables. La prevención de diferentes patologías como por ejemplo enfermedades cardiovasculares (Estruch *et al.* 2013) o cáncer (Escrich *et al.* 2011) ha sido asociada al consumo de aceite de oliva virgen (AOV).

2.1 Aceite de oliva virgen

El aceite de oliva virgen (AOV) está compuesto en su mayoría por triglicéridos (entre un 98% y un 99%) constituyendo la fracción saponificable o mayoritaria. El resto (entre un 1% y un 2%) lo forman alrededor de unas 230 sustancias muy diversas, parte de las cuales constituyen la fracción insaponificable o minoritaria (figura 6).
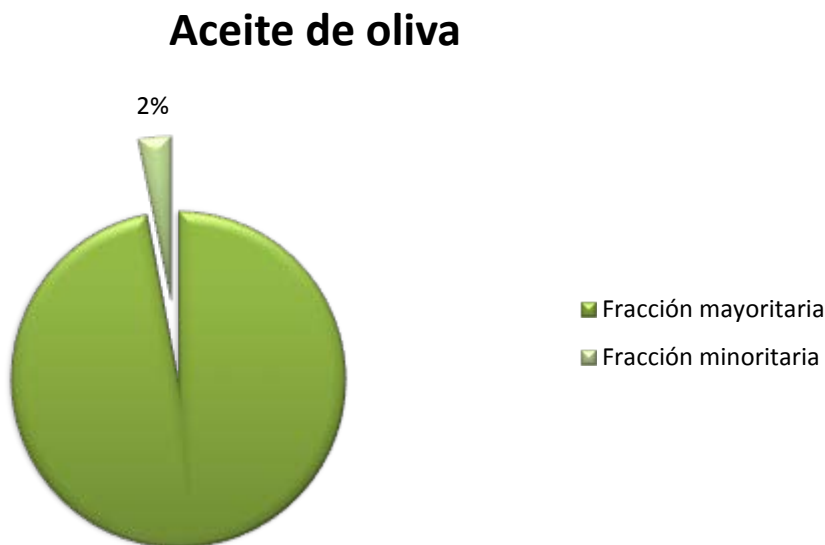
Aceite de oliva

Figura 6. Porcentajes relativos a la composición del aceite de oliva.

Más del 90% del AOV se forma en el mesocarpo de la drupa del fruto del olivo (*Olea europaea* L.). El AOV se obtiene de aceitunas procesadas mediante métodos físicos y sin añadir ningún tipo de aditivo o conservante. Es la única grasa comestible que puede ser consumida como un zumo natural extraído de un fruto, la aceituna. Cabe destacar que una vez que el AOV es refinado, la mayoría de componentes minoritarios disminuyen o desaparecen.

2.1.1. Composición del aceite de oliva virgen

2.1.1.1. Fracción mayoritaria

Triacilglicéridos

Están compuestos por una molécula de glicerol y tres ácidos grasos.

Ácidos grasos libres

Los ácidos grasos están constituidos por largas cadenas formadas por átomos de carbono. Los ácidos grasos se pueden clasificar en ácidos grasos saturados (SFA; satured fatty acids) caracterizados por no poseer ningún doble enlace ni ningún tipo de grupo funcional a lo largo de la cadena, teniendo toda la cadena saturada con átomos de hidrógeno; ácidos grasos monoinsaturados (MUFA; monounsatured fatty acids) dónde dos átomos de carbono se encuentran unidos por un doble enlace *cis* y en ácidos grasos poliinsaturados (PUFA; polyunsatured fatty acids) cuyas cadenas de carbono poseen dos o más enlaces dobles *cis*. Los tres tipos se encuentran presentes en el AOV, pero con un claro predominio de los MUFA, que representan entre el 55-83% de los ácidos grasos totales y cuyo principal representante, presente en el AOV, es el ácido oleico (18:1 ω9). La concentración de ácido palmítico y ácido esteárico (ambos SFA) ronda entre el 8-25%; y de ácido α-linolénico y ácido linoleico (PUFA) el 3-21% en el AOV. Los porcentajes varían dependiendo del clima, el estado de madurez del fruto, de las condiciones de cultivo y sobre todo de la variedad vegetal (López *et al.* 2014).

2.1.1.2. Fracción minoritaria

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 2010).

Compuestos fenólicos

Son los que confieren el sabor amargo al AOV (Beltrán *et al.* 2000) y los que lo protegen de la oxidación (Baldioli *et al.* 1996). Son estructuras orgánicas que presentan anillos aromáticos funcionalizados con grupos OH, que les confieren solubilidad en agua. Dependiendo de su estructura concreta se reparten entre el agua y el aceite. Esta solubilidad parcial en aceite y agua hace que el proceso de elaboración influya notoriamente en las concentraciones finales de estas sustancias en el aceite. Además su carácter ligeramente ácido les hace reaccionar con las disoluciones alcalinas para formar sales (mas solubles aún en agua) por lo que los procesos de neutralización del aceite de oliva refinado los elimina del mismo. Su

concentración depende de muchos factores entre los que habría que destacar la variedad de planta de la que se obtienen y el proceso seguido en la elaboración del aceite. Las concentraciones en las que están presentes en el AOV varían entre 50 y 500 mg/kg expresado en cantidad de ácido cafeico. En este grupo se encuentran compuestos como el tirosol, hidroxitirosol, ácido homovainíllico, ácido caféico, pinoresinol, acetoxipinoresinol e hidroxipinoresinol entre otros.

Hidrocarburos

El hidrocarburo más abundante en el AOV es el escualeno (entre 800-12000 mg/kg), aproximadamente un 90% del total de hidrocarburos presentes en el AOV.

Tocoferoles

Son compuestos que otorgan estabilidad al aceite, y poseen propiedades antioxidantes. El más abundante es el α-tocoferol (vitamina E) que constituye el 95% del total, el 5% restante está formado por β-tocoferol y γ-tocoferol.

Alcoholes alifáticos

Gracias a estos alcoholes alifáticos se han podido diferenciar entre aceite de oliva virgen y de orujo (Comisión Europea 1991). 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 AOV son las C40, C42, C44 y C46, sin llegar a superar los 350 mg/kg en total.

Fitoesteroles

Los principales son el β-sistosterol (75-90 % del total), el Δ-5-avenasterol (5-20%), campesterol (1-4%), el sitostanol (0,7-2,5%) y el estigmasterol (0,5-2%). Se presentan libres o esterificados con ácidos grasos, en conjunto suponen entre el 0,125-0,250% del peso total de un aceite de oliva virgen. Los valores son mucho mayores en aceites de orujo.

Carotenoides

Son sustancias coloreadas que actúan como pigmentos del AOV. Las más importantes son la luteína y el β-caroteno. La cantidad en el AOV oscila entre 1-20

ppm (partes por millón). Este contenido se ve afectado según la variación estacional, disminuye progresivamente a medida que avanza la recolección.

Clorofilas y feofitinas

Junto con los carotenoides son los responsables del color del AOV. 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 AOV. 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 2010).

Dialcoholes triterpénicos

Grupo formado principalmente por el uvaol y el eritrodiol cuyas concentraciones en el AOV son del orden de 8,15 a 85,05 mg/kg (Allouche *et al.* 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 AOV. 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 AOV 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 *et al.* 2009).

2.1.2. Propiedades bioactivas de los componentes del AOV

Tras la ingestión de AOV, los ácidos grasos y triacilgliceroles pasan al torrente sanguíneo formando parte de los quilomicrones. Y una vez que los quilomicrones llegan a la superficie de las células, son hidrolizados por ciertas lipasas dando lugar a ácidos grasos y a moléculas de glicerol. Los ácidos grasos pasan por difusión al citoplasma de la célula, dónde serán utilizados para conformar moléculas que formarán parte de la biogénesis de esa célula, por ejemplo entrarán a formar parte de la membrana celular.

La membrana celular contiene cientos de lípidos diferentes, clasificados en 3 tipos: glicerofosfolipidos, enfingolípidos y esteroles (colesterol en animales). Los glicerofosfolípidos están conformados, entre otros componentes, por gliceroles y ácidos grasos. La diferencia de ácidos grasos y sus enlaces van a determinar la complejidad de los glicerofosfolípidos. El esqueleto lipídico de los enfingolípidos está compuesto por una molécula de ceramida (esfingosina unida a un ácido graso mediante un enlace amida).

Se requiere una considerable parte del genoma para sintetizar, metabolizar y regular el conjunto de ácidos grasos que se sintetiza *de novo*, así como para incorporar aquellos procedentes de la dieta en la biogénesis de la membrana. Pero se necesitan más estudios para aclarar el papel que desempeñan todas las moléculas implicadas y las rutas que utilizan.

Parece que la incorporación del ácido oleico en los fosfolípidos de la membrana celular regula la estructura de la membrana y sus propiedades biofísicas. De hecho la incorporación de este ácido graso y no otro reduce los giros de moléculas dentro de la membrana, ayuda a mantener el nivel de hidratación e incrementa la fluidez de la membrana, características que son beneficiosas para la promoción de la salud, e incluso previenen ante el riesgo de padecer enfermedades relacionadas con anormalidades en el tipo y/o abundancia de los lípidos de la membrana celular (López *et al.* 2014).

El principal hidrocarburo del AOV es el escualeno, el cual entra a formar parte del metabolismo del colesterol en animales, siendo precursor directo del lanosterol. Este escualeno es introducido en la membrana celular donde juega un papel fundamental en el mantenimiento del gradiente electroquímico encargado del transporte de iones a través de la membrana. Se ha visto que gracias a él se incrementa la polaridad y las interacciones hidrofóbicas dentro de la membrana, facilitando la regulación funcional de proteínas encargadas del movimiento de líquidos a través de la membrana. También se ha comprobado su acción ante señales químicas, físicas, bacterianas o

estresantes del exterior, protegiendo la superficie de la piel. De hecho se ha estudiado su acción protectora ante la rotura del ADN tanto en células normales de mama como en células epiteliales humanas (Warleta *et al.* 2010; López *et al.* 2014).

La luteína y el caroteno son protectores ante el daño oxidativo en la retina, así como protectores ante la degeneración macular debida a la edad. También se han descrito efectos protectores ante las cataratas, así como la fibrilación atrial y fracturas osteoporóticas. Compuestos fenólicos, lignanos y demás compuestos minoritarios del AOV poseen un papel importante en la constitución de membranas celulares y en sus funciones, características que les otorga un papel protector frente a la aparición de diversas funciones relacionadas con el metabolismo lipídico de la célula (López *et al.* 2014).

3. Compuestos triterpénicos presentes en el AOV: ácido oleanólico, ácido maslínico, uvaol y eritrodiol

Los principales componentes del grupo de triterpenos que podemos encontrar en el AOV son el ácido oleanólico, ácido maslínico, uvaol y eritrodiol.

Las concentraciones en la que se encuentran en el AOV varía según factores genéticos, siendo mayores en las variedades Lechín de Granada, Dolce Agogia, Cornicabra y Salonenque (con valores comprendidos entre los 197 mg/kg y los 127 mg/kg) y menores en las variedades Pico Limón de Grazalema, Genovesa, Pajarero y St. George Greys (con un contenido medio de 27mg/kg) (Allouche *et al.* 2009).

El ácido oleanólico (3beta-Hydroxyolean-12-en-28-oic acid), el ácido maslínico ((2|A,3|A)-2,3-dihydroxyolean-12-en-28-oic acid), el uvaol ((3beta)-Urs-12-ene-3,28-diol) y el eritrodiol ((3beta)-Olean-12-ene-3,28-diol) se sintetizan en el olivo mediante la vía acetato/mevalonato citoplasmática compartiendo un precursor común, el 2-3-oxidoescualeno (figura 7).
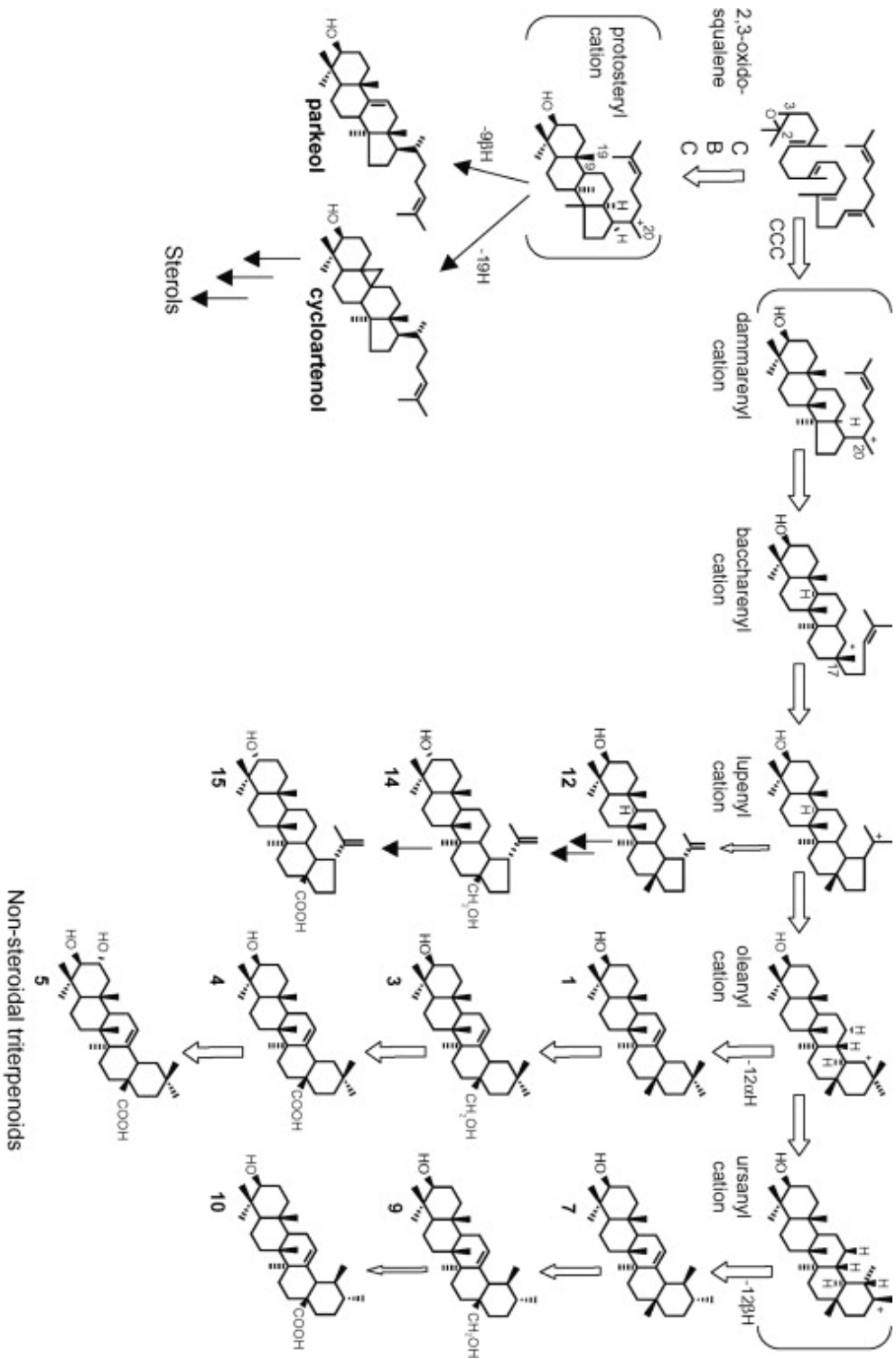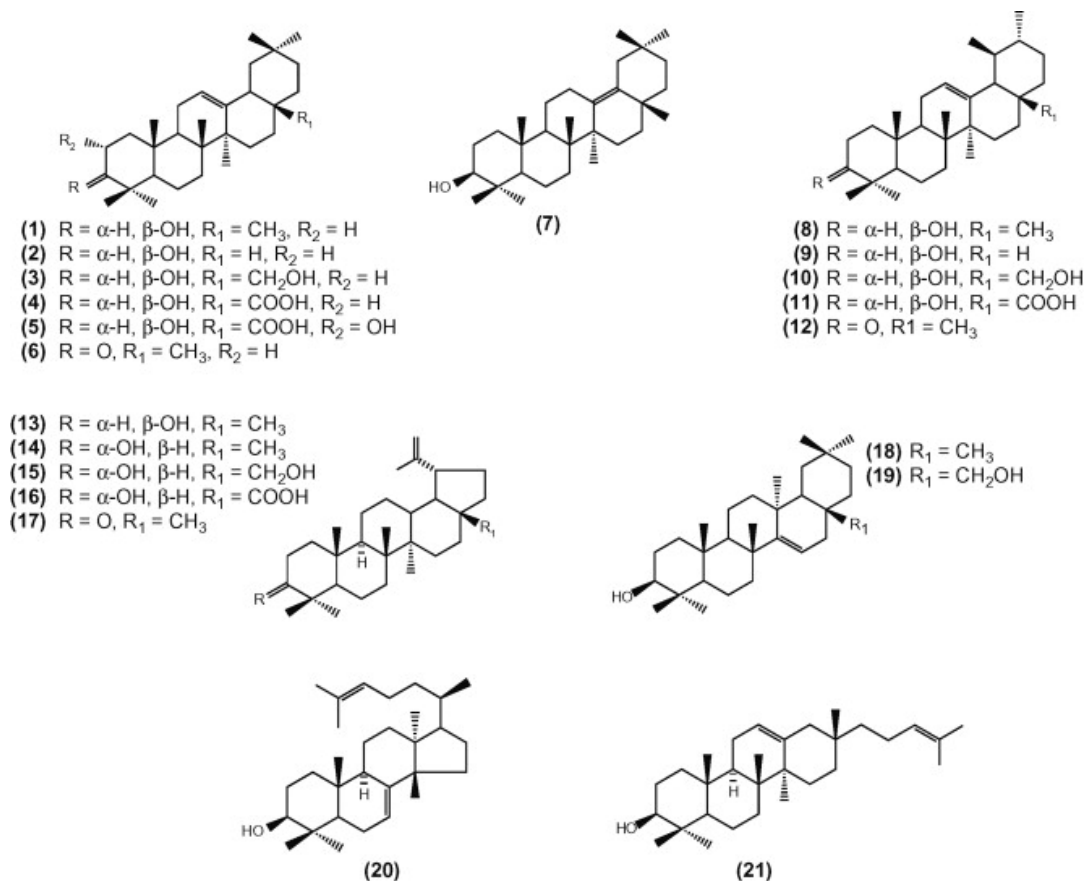
Figura 7. Síntesis y estructura de los compuestos triterpénicos en el desarrollo del fruto del olivo (Stiti *et al.* 2010). CBC: conformación silla-bote-silla del 2,3-oxidoescualeno. CCC: conformación triple silla del 2,3-oxidoescualeno. Compuestos del tipo oleanano (**1**–**7**) (β-amirina **1**, 28-nor-β-amirina **2**, eritrodiol **3**, ácido oleanólico **4**, ácido maslínico **5**, β-amirona **6** y β-amirin **7**), del tipo ursano (**8**–**12**) (α-amirin **8**, 28-nor-α amirin **9**, uvaol **10**, ácido ursólico **11** y α-amirona **12**), del tipo lupano (**13**–**17**) (lupeol **13**, 3-*epi*-lupeol **14**, 3-*epi*-betulin **15**, 3-*epi*-ácido betulínico **16** y lupenona **17**) y del tipo taraxeno (**18**–**19**) (taraxerol **18**, taraxer-14-ene-3β,28-diol **19**), así como esqueletos carbonados de tipo eufano (butyrospermol **20**) y de tipo bacarano (bacar-12,21-dien-3β-ol **21**).

Tanto el eritrodiol como los ácidos oleanólico y maslínico proceden del oleanano, mientras que el uvaol procede de un esqueleto carbonado del tipo ursano.

La síntesis de estos compuestos dentro del fruto se lleva a cabo con la maduración del mismo, de manera que los contenidos de uvaol y eritrodiol aumentan en las primeras semanas tras el desarrollo del fruto (cuando la lignificación del endocarpo del fruto ha terminado) y después van desapareciendo con el paso de las semanas a medida que aumenta el contenido en ácido oleanólico y maslínico, alcanzando su

máximo en las semanas 28-30 después del desarrollo del fruto (componiendo el 98% del total de triterpenoides) (Stiti *et al.* 2010).

El contenido de triterpenos en las aceitunas de mesa negras y verdes abarca desde los 460 a los 1470 mg/kg. La pulpa de las aceitunas negras naturales (sin tratamiento con NaOH) presenta concentraciones mayores a 2000 mg/kg. Mientras que la hoja contiene grandes cantidades de ácido oleanólico (3.0-3.5%) seguido del ácido maslínico y en menor medida de uvaol y eritrodiol (Sánchez-Quesada *et al.* 2013).

Aun así, dependiendo tanto del tipo de cultivo como del proceso y manejo de extracción del aceite de oliva, la cantidad en triterpenos variará. De hecho los aceites con mayor contenido en triterpenos son los aceites de orujo de oliva y los de menor contenido, los aceites de oliva virgen (Pérez-Camino *et al*. 1999).

3.1. Biodisponibilidad de los triterpenos de Olea europaea sp.

Actualmente encontramos pocos artículos que revelen la cantidad disponible en las células tras la ingesta de aceite de oliva o del producto aislado.

<u>Ácido oleanólico</u>

Estudios en ratas demuestran que tras la ingesta de 50 mg/kg, la concentración máxima de ácido oleanólico encontrada fue de 0,29 ± 0,26 µM a los 21 ± 17 min (Jeong *et al.* 2007). Según este estudio la biodisponibilidad oral absoluta fue de 0,7%. En humanos las concentraciones plasmáticas encontradas después de una dosis única oral de 40 mg fue de 26,5 ± 15 nM a las 5,2 ± 2,9 h (Song *et al.* 2006). En la tabla 2 se enumera el contenido encontrado en órganos y plasma en ratones suplementados con 5% de ácido oleanólico.

<u>Ácido maslínico</u>

En ratas, tras una ingesta única oral de 50mg/kg, fue encontrado en sangre tras 10 min desde la ingesta y seguía en sangre 60 min después (Lozano-Mena *et al.* 2012). Tras 24 h desde la dosis oral de ácido maslínico (administración de 10 mg/kg) las concentraciones en el plasma de las ratas fueron de 10,8 ± 2,2 nM (Sánchez-González *et al.* 2013). La tabla 2 muestra el contenido en ácido maslínico encontrado en tejidos de diferentes órganos y en plasma de ratones suplementados con un 5% del compuesto.

<u>Uvaol y eritrodiol</u>

Por el momento no existen referencias acerca de la biodisponibilidad de estos triterpenos en animales.

	plasma	cerebro	corazón	hígado	riñón	colon	vejiga
OA, 4 semanas	-	0.5 ± 0.1	2.7 ± 0.6	5.9 ± 0.8	3.0 ± 0.3	3.4 ± 0.7	1.2 ± 0.5
OA, 8 semanas	0.55 ± 0.08	1.7 ± 0.2	4.2 ± 0.8	10.3 ± 1.4	5.5 ± 0.5	6.0 ± 1.0	3.7 ± 0.4
MA, 4 semanas	-	0.8 ± 0.3	2.7 ± 0.5	5.4 ± 0.6	3.6 ± 0.6	3.5 ± 0.7	1.0 ± 0.2
MA, 8 semanas	0.47 ± 0.07	1.6 ± 0.5	3.7 ± 0.7	9.6 ± 0.9	6.5 ± 0.8	7.0 ± 0.8	1.9 ± 0.5

Tabla 2. Contenido de ácido oleanólico (OA) y ácido maslínico (MA) en plasma (μg/mL) o distintos tejidos (μg/g) en ratones alimentados con OA o MA al 0,5% durante 4 u 8 semanas (Yin *et al.* 2012).

3.2. Propiedades bioactivas de los triterpenos en cáncer de mama

Ácido oleanólico

El ácido oleanólico parece poseer efectos antitumorales en distintos tipos de cáncer. Induce apoptosis y detiene el ciclo celular en carcinomas hepatocelulares, en líneas tumorales humanas de páncreas (Panc-28), líneas tumorales de pulmón, adenocarcinoma de pulmón y células de melanoma (B16F10) entre otras (Sánchez-Quesada *et al.* 2013).

Pero en cáncer de mama hay pocos estudios que describan su acción antitumoral. Allouche *et al*. describieron la acción antitumoral de este compuesto en la línea tumoral humana de mama MCF7 (Allouche *et al.* 2011), así como Liu *et al*. analizaron el mecanismo de acción por el que el ácido oleanólico ejercía esa actividad antitumoral en MCF7, una inhibición de la glucólisis aeróbica (Liu *et al.* 2014). Estos mismos efectos (tanto en MCF7 como en la línea epitelial MDA-MB-231) han sido observados con extractos de la planta *Wrightia tomentosa* que contenían ácido oleanólico (Chakravarti *et al.* 2012).

Este compuesto también presenta actividad antimetastásica, inhibiendo la migración en la línea tumoral humana de mama MDA-MB-231 (Elsayed *et al.* 2014).

En la actualidad ya se han realizado ensayos para probar la acción de análogos a este compuesto que hipotéticamente poseen mayor actividad antitumoral en cáncer de mama *in vitro* (Elsayed *et al.* 2014; Lisiak *et al.* 2014; Mallavadhani *et al.* 2014).

Ácido maslínico

El efecto antitumoral que produce el ácido maslínico ha sido ampliamente estudiado en cáncer de colon *in vitro* e *in vivo*. También ha sido descrita una actividad antimetastásica en líneas tumorales de próstata humanas (DU145) mediante la inhibición de factores como el VEGF, las MMPs o el incremento de expresión de las proteínas Bid y Bax (Sánchez-Quesada *et al.* 2013).

En cáncer de mama la bibliografía que describe la acción del ácido maslínico se basa en ensayos realizados con extractos de plantas que inhiben el crecimiento e inducen apoptosis en las células tumorales de mama MCF7 y SKBR3 (Plastina *et al.* 2012) y estudios del componente aislado que posee actividad antiproliferativa en distintas líneas tumorales, entre ellas MCF7 (He y Liu 2007).

Uvaol

Los efectos en cáncer que posee este compuesto se describen por primera vez en 1976 en la línea leucémica P-388 (Trumbull *et al.* 1976).

Es Saady *et al*. describieron los efectos inhibitorios del crecimiento en MCF7 del uvaol, aislado de la planta *Calluna vulgaris* (Es Saady *et al.* 1995). Tras ellos, más estudios realizados también en MCF7 demuestran los efectos antiproliferativos de este compuesto y su acción en el ciclo celular de la célula tumoral de mama (Allouche *et al.* 2011).

Eritrodiol

El eritrodiol posee actividad antitumoral en cáncer de piel y en líneas tumorales humanas y de ratón con efectos proapoptóticos (Sánchez-Quesada *et al.* 2013).

En líneas tumorales de mama (MCF7) eritrodiol muestra un efecto citotóxico dependiente de dosis y de tiempo, así como efecto proapoptótico y daño oxidativo al ADN de la célula tumoral (Allouche *et al.* 2011).

HIPÓTESIS Y OBJETIVOS

Hipótesis

Las evidencias científicas publicadas por diferentes autores sugieren que determinados tipos de tumores, el de mama entre ellos, son menos frecuentes en aquellos países donde se consume habitualmente aceite de oliva virgen.

Algunos triterpenos, como el uvaol, eritrodiol, ácido maslínico y ácido oleanólico, presentes en los aceites de oliva vírgenes podrían ser los que, al menos parcialmente, le confieran un papel protector a esta grasa frente al desarrollo del cáncer de mama en humanos.

Objetivos

Partiendo de esta hipótesis se plantearon los siguientes objetivos:

➢ Determinar el efecto antitumoral del ácido oleanólico, ácido maslínico, uvaol y eritrodiol sobre células humanas de cáncer de mama procedentes de una línea celular altamente metastásica (MDA-MB-231).

➢ Determinar el efecto protector ante el daño oxidativo inducido en células epiteliales mamarias humanas procedentes de una línea celular no tumoral (MCF10A) imputados a la acción del ácido oleanólico, ácido maslínico, uvaol y eritrodiol.

➢ Determinar el efecto antiinflamatorio del ácido oleanólico, ácido maslínico, uvaol y eritrodiol utilizando una línea celular monocitaria humana (THP-1) diferenciada a macrófago de tipo M1.

TRABAJO EXPERIMENTAL Y RESUMEN GLOBAL DE LOS RESULTADOS

Trabajo experimental y resumen global de los resultados

   1.Oleanolic acid selectivity inhibits proliferation of highly invasive human breast cancer cells and protects against oxidative DNA damage in normal mammary epithelial cells.

Trabajo enviado a la revista "Nutrients".

- Índice de Impacto: 3.148
- Categoría perteneciente: Nutrición y Dietética
- Cuartil en la categoría: Q2
- Rango en la categoría (2013): 23/78
- Fecha de envío: 22/09/2014

Oleanolic acid selectivity inhibits proliferation of highly invasive human breast cancer cells and protects against oxidative DNA damage in normal mammary epithelial cells.

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Abstract

Virgin olive oils contain a wide variety of minor compounds, including both oleanolic (OA) and maslinic (MA) acids which are pentacyclic triterpenes that possess interesting beneficial properties. In fact, high consumption of virgin olive oil has been associated with a low incidence of breast cancer. However, at the present there is no study about the potential chemopreventive effects of both pentacyclic triterpenes in human breast cancer. In the present paper, we try to elucidate if both compounds possess breast cancer chemopreventive activities. A highly invasive human breast cancer cell line (MDA-MB-231), a minimally invasive human breast cancer cell line (MCF7) and an immortalized non-tumorigenic human breast epithelial cell line (MCF10A) were used for determination of in vitro effects of both, OA and MA. To this end, we measured the following: cytotoxic activity, proliferation rate, cell cycle arrest, apoptosis, oxidative stress through ROS quantification, catalase activity and DNA damage. OA inhibited proliferation in the highly invasive MDA-MB-231 and increased oxidative stress MDA-MB-231. Besides, OA decreased oxidative stress and oxidative

damage to DNA in the non-tumorigenic breast cell line MCF10A. MA enhanced oxidative stress in MCF7 cancer cell line and inhibited proliferation in MDA-MB-231 cell line. In MCF10A cells, MA promotes apoptosis and increased the oxidative damage induced to DNA. The data obtained suggest that, OA has the potential to act as a chemopreventive in human breast cancer and also, it has the capacity to arrest proliferation of the metastatic breast cancer cells.

Keywords: virgin olive oil, oleanolic acid, maslinic acid, breast cancer, MCF7, MDA-MB-231, MCF10A

Introduction

The triterpenoids are natural compounds widely distributed in many plants and foods. Virgin olive oils contain a wide variety of minor compounds, including both oleanolic and maslinic acids [1] which are pentacyclic triterpenes that possess interesting beneficial properties. The traditional Mediterranean diet, characterized by high consumption of virgin olive oil as main fat source, has been associated with a low incidence of breast cancer [2]. Current knowledge highlights the role of minor compounds from virgin olive oil in the prevention of certain cancers, including breast cancer [3-6]. Interestingly, it has been described that oleanolic acid and maslinic acid possess cardioprotective effects [7,8], anti-inflammatory effects [9,10], and antitumor properties in human prostate cancer cells [11], hepatocellular carcinoma cells [12], human pancreatic cells [13] and colon cancer cells among others [14,15]. However, so far there is no study about the potential chemopreventive effects of both pentacyclic triterpenes in human breast cancer. We hypothesized that, at least in part, the chemopreventive effects of virgin olive oil may be due to the biological actions exerted by these compounds. In order to demonstrate this hypothesis, we have used for an *in vitro* study the following well-characterized human cell lines: non-tumorigenic MCF10A human breast epithelial cells (oestrogen receptor-negative); highly invasive MDA-MB-231 human breast cancer cells (oestrogen and progesterone receptor-negative); and finally, minimally invasive MCF7 human breast cancer cells (oestrogen and progesterone receptor-positive).

Materials and Methods

Chemicals

Oleanolic acid (OA) CAS [508-02-1] (purity ≥97%) was purchased from Extrasynthese (Genay, FRANCE). Maslinic acid (MA) CAS [4373-41-5] (purity ≥98%) was obtained from Cayman Chemical (Ann Arbor, MI, USA). The following were purchased from Sigma-Aldrich Co. (St Louis, MO, USA): Hepes solution; Sodium

Pyruvate solution; Non-Essential Amino Acids mixture 100× (NEAA); 2,7-dichlorofluorescin diacetate (DCFH-DA) CAS [4091-99-0] (purity ≥97%); 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%); phosphate buffered saline (PBS); (S)-(+)-camptothecin (CPT) CAS [7689-03-4] (purity ≥90%) and Triton X-100. Foetal Bovine Serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, AUSTRIA). TrypLE Express, HuMEC ready medium, Minimum essential medium with Eagle's salts (MEM) and Phenol-Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from Gibco® Life Technologies Ltd (Paisley, UK). Methanol dry (max 0,005%) and ethanol absolute PRS was purchased from Panreac Quimica S.L.U. (Barcelona, SPAIN). CellTiter-Blue® Cell Viability Assay was obtained from Promega Corporation (Madison, WI, USA). Phosphate buffered saline (1X, Dulbecco's) (PBS) was purchased from Applichem GmbH (Gatersleben, GERMANY). Culture plates were obtained from Starlab (Hamburg, GERMANY). The PI/RNase Staining Buffer kit was obtained from BD Biosciences Pharmigen (San Diego, CA, USA). Annexin-V FITC kit was purchased by Miltenyi Biotec (Cologne, GERMANY). The Comet Assay kit was obtained from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD, USA). The Catalase Assay kit was purchased by Merck KGAA (Darmstadt, GERMANY).

Cell culture and treatments

Highly invasive MDA-MB-231 (ATCC® Number: HTB-26 ™) human breast cancer cells (oestrogen and progesterone receptor-negative), minimally invasive MCF7 (ATCC® Number: HTB-22 ™) human breast cancer cells (oestrogen and progesterone receptor-positive), and immortalized non-tumorigenic MCF10A (ATCC® Number: CRL-10317 ™) human breast epithelial cells (oestrogen receptor-negative), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Breast cancer 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 grown in HuMEC Ready Medium. All cell lines were maintained at 37 ºC in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments.

Except for the assays which specified the opposite, cells were treated with 0,1 μM, 1 μM and 10 μM of oleanolic acid (OA) and maslinic acid (MA) for 4 h.

Cytotoxicity Assay

Cell survival, measured as the cellular growth of treated cells *versus* untreated controls, was carried out in MCF10A, MCF7 and MDA-MB-231 using an XTT-based assay according to Scudiero et al. [16] with some modifications. Briefly, cells were seeded into 96-well culture plates in a total volume of 100 μL per well (5 x 10³

cells/well for MDA-MB-231 and MCF7 and 2,5 x 10^3 cells/well for MCF10A). After overnight incubation to allow cell attachment, 100 µL of fresh medium was added containing increasing concentrations from 0,001 µM to 100 µM of OA and MA for 24 h. Thereafter, cells were incubated with XTT in Phenol-Red free RPMI medium for 3 h at 37 °C with 5% CO_2, and absorbance was measured at 450nm wavelength (620nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

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

Where A is the difference in absorbance between optical density units ($A = OD_{450} - OD_{620}$). All measurements were performed in quadruplicate and each experiment was repeated at least three times. As a vehicle control, cells were treated with Et-OH at the highest concentration of OA and MA used.

Cell proliferation assay

Cell proliferation, measured as the cellular growth of treated cells versus untreated controls, was carried out using CellTiter-Blue Cell Viability Assay. Briefly, cells were seeded into 96-well culture plates at 2 x 10^3 cells/well for MCF7, 1 x 10^3 cells/well for MDA-MB-231 and 0,5 x 10^3 cells/well for MCF10A. After overnight incubation to allow cell attachment, medium was removed and replaced with fresh medium containing OA and MA from 0,01 µM to 100 µM. Plates were incubated for 24, 48 or 72 h followed by a 72 h, 48 h and 24 h proliferation period (incubation with fresh medium without OA or MA) respectively. At these three time points, plates were incubated with CellTiter-Blue Cell Viability for 3 h at 37 °C with 5% CO_2 and the relative fluorescence units were measured in a plate reader (TECAN GENios Plus) (Ex. λ_{485}/Em. λ_{595}, Gain 60). Viability was calculated using the formula:

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

Where A are the relative fluorescence units for each sample. All measurements were performed in triplicate and each experiment was repeated at least three times. As a vehicle control, cells were treated with Et-OH at the highest concentration of OA and MA used.

Cell cycle assay

Cells were seeded in 12-well culture plates (1 x 10^5 cells/well for MDA-MB-231 and MCF7; and 0,5 x 10^5 cells/well for MCF10A) and incubated overnight to allow cells attachment. Then, cells were treated with 0,1 µM, 1 µM and 10 µM of OA and MA for 24 h; cells were harvested with TrypLE Express and washed with 1x PBS (Ca2+/Mg2+ free) (300 xg 10 min at 4 °C). After, cells were fixed with cold 70% ethanol and stored at -20 °C for at least 24 h. Subsequent to propidium iodide

labelling (PI/ RNase Staining Buffer), cells were analysed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). The FlowJo program (v5.7.2) was used to calculate the percentage of cells in G0/G1, S and G2/M phases. Each 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). Briefly, cells were seeded in 12-well culture plates (1 x 10^5 cells/well for MDA-MB-231 and MCF7; and 0,5 x 10^5 cells/well for MCF10A) and incubated overnight to allow cell attachment. After cells exposure to OA and MA for 24 h at 0,1 µM, 1 µM and 10 µM, cells were harvested with TrypLE Express, washed twice in cold 1x PBS (Ca2+/Mg2+ free) (300 xg 10 min at 4 °C) and resuspended in 100 µL of Annexin Binding Buffer. Cells were stained with 5 µL Annexin V-FITC and 2 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.

Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured after 4 h of treatment with OA and MA at a range from 0,0001 µM to 100 µM, using the cell-permeable fluorescent probe, 2,7-dichlorofluorescin diacetate (DCFH-DA), as previously described by Warleta et al. [5] with some modifications. Briefly, cells were seeded on 96-well plate (5 x 10^3 cells/well for MDA-MB-231 and MCF7 and 2,5 x 10^3 cells/well for MCF10A) and after incubation with treatments, DCFH-DA (100 µM) was added for 30 min at 37 °C with 5% CO_2. Cells were then 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:

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

Where $F_{t=0}$ is the fluorescence at $t = 0$ min and $F_{t=30}$ the fluorescence at $t = 30$ min. It has been described that the addition of H_2O_2 increases oxidative stress in cultured cells and directly damage DNA [17]. To evaluate the protective capacity of OA and MA 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).

Determination of catalase (CAT) activity

Cells were seeded into a 6-well plate at 0.5×10^6 cells/mL for MCF10A , MDA-MB-231 and MCF7. Cells were incubated overnight for cells attachment. After that, medium was changed by fresh medium with OA and MA. The assay was made according to manufacturer's protocol, for determination of the enzymatic activity of catalase.

Alkaline single-cell gel electrophoresis (Comet Assay)

Cells were seeded into 12-well plate (1×10^5 cells/well for MDA-MB-231 and MCF7 and 0.5×10^5 cells/well for MCF10A) and incubated overnight for cells attachment. Then cells were treated with OA and MA. After, cells were scraped and washed twice (300 xg 10 min, 4 ᵒC) with cold 1X PBS (Ca2+/Mg2+ free). They were resuspended in 1 mL of cold 1x PBS. In order to evaluate OA and MA ability to protect against oxidative DNA damage, cells were exposed for 10 min to 50 µM H_2O_2 at 4 ᵒC. After that, the comet assay was performed according to Warleta et al. [5].

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). Twenty five cell images were randomly characterized per sample using 20x magnifications. 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.

Olive_TM = [(tail (mean) – head (mean)) x tail (% DNA)] / 100

Statistical analysis

Results are displayed as the mean of at least three independent experiments (±SEM), and results are expressed as a percentage relative to the untreated control, which was set 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.

Results

Cytotoxicity effects

Cell viability of untreated and treated cells with OA and MA was determined by the XTT assay, which is based on the ability of live cells to cleave the tretazolium ring, thus producing formazan, which absorbs at 570 nm [16]. The absorbance of cells

treated with vehicle control (Et-OH at 1%) was determined too, but not statistical difference was found respect to untreated control (data not shown). Results are expressed as percentage of cell survival respect to untreated control, which was set as 100%.

For MCF10A, OA and MA at 10 and 100 μM promoted cell death (cell survival is 83% and 13% for OA and 9% and 13% for MA, respectively) (Fig 1A). For MCF7, MA induced a strong cytotoxic effect at 100 μM (8%) (Fig 1B). MDA-MB-231 treated with the two acids showed a marked cytotoxic effect for OA and MA at 100 μM (68% and 17% respectively). The concentrations between 0,01 μM and 10 μM of MA appeared to promote cell survival (Fig 1C).

In the normal epithelial cell line, both compounds were cytotoxic at the highest concentrations. But for MCF7, which is a multi-drug resistance cancer cell line, only MA was capable of produce mortality. OA did not produce cytotoxicity in MCF7, or at least statistically significant. Our results agree with Shan et al. who showed that OA had no a strong effect inhibiting the growth of MCF7 [18]. In MDA-MB-231, other studies about extraction of different plants (which contain OA) described antiproliferative effects [19,20]. Ponou et al. showed that OA isolated did not produce any cytotoxicity at maximum concentration of 200 μM [21], while we observed cytotoxicity at 100 μM.

Effects on proliferation

Proliferation was determined by CellTiter-Blue Cell Viability, which is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. Vehicle control was used (Et-OH at 1%) for determination of fluorescence and not statistically difference was found respect to untreated control (data not shown). Results are expressed as percentage of cell survival respect to untreated control, which was set as 100%.

MA at 10 and 100 μM showed antiproliferative effects for MCF10A at 24, 48 and 72 h (10%, 38% and 11% of cell survival for 10 μM and 9%, 10% and 11% for 100 μM respectively) (Fig 2). For MCF7 only at 100 μM this compound showed inhibition of proliferation statistically significant at all times assayed (5%, 5% and 19% of cell survival respectively) (Fig 3). An inhibitory effect of proliferation was exerted by MA in a dose dependent manner with 48 and 72 h of treatment in MDA-MB-231 (Fig 4).

In MCF10A, OA inhibited proliferation at 10 and 100 μM after 48 and 72 h of treatment (~65% and 9% of cell survival in both times) (Fig 2B-2C). In MDA-MB-231, OA exerted an inhibition of proliferation in a dose-dependent manner after the treatment exposure times (Fig 4).

According to this, OA and MA at 10 and 100 µM inhibited proliferation in normal breast cells. But at lower concentrations than these, they appeared increase the proliferation of the normal breast cells along the time. For MCF7, MA only at 100 µM was capable of reducing the proliferation. Interestingly, OA and MA were able to inhibit proliferation in a dose-dependent manner with all the time exposures assayed in the highly invasive breast cancer cell line (MDA-MB-231).

Effects on cell cycle

Cell cycle was determined by flow cytometry. Results are expressed as percentage of cells in the different phases of cell cycle.

For MCF10A, OA showed an increase in G0/G1 phase at 10 µM respect to control, and a decrease in G2/M phase. Otherwise, MA showed a high increase in sub G0/G1 phase at 10 µM (65%) respect to control (0,4%) and consequently, a decrease in the other phases. At 10 µM, both compounds affect cell cycle of MCF10A (table 1). Therefore, depend on the concentration used these compounds could damage the cell. The caution with the concentration assayed with these triterpenes in different experiments was discussed recently by our group [22], and our results showed that high concentrations of these compounds can promote cell death in normal breast cells.

For MDA-MB-231, OA exerted a decrease in G0/G1 respect to control and an increase in G2/M at 0,1 µM. At 10 µM, OA increased G2/M phase respect to control. MA did not show any difference statistically significant in MDA-MB-231 (table 1) or MCF7 (data not shown).

According to these results, it seems that MA affect cell cycle of MCF10A cells, increasing the SubG0/G1 ratio. This increase could be due to proapoptotic effects. For assess this apoptotic effect, our group studied apoptosis of these compounds in the three breast cell lines.

Analysis of Apoptosis

Apoptosis was assessed by flow cytometry. Percentage of living, apoptotic and necrotic cells are represented respect to the total, which was set as 100% (table 2).

For MCF10A, OA at 10 µM had a higher percentage of apoptotic cells respect to control. MA at 10 µM increased the rate of apoptotic cells. For MDA-MB-231 not statistically significant differences were found, but OA at 1 µM showed a slight increase in apoptotic cells rate (table 2). MA treatment in MCF7 did not show any difference respect to control (data not shown).

Thereby, MA and OA at the highest concentration caused apoptosis in MCF10A cells, while concentrations lower than 10 µM appeared not promote apoptosis. However, in both breast cancer cell lines neither OA nor MA produced a dramatically increase of

apoptosis, only OA 1 μM increased softly the apoptotic ratio in MDA-MB-231. This soft increase could correspond with the proliferation observed, where OA decreased proliferation in a dose-dependent manner along the time exposure.

Effects on intracellular ROS level

In MCF10A, MCF7 and MDA-MB-231, DCFH-DA probe was used for asses the ROS levels by fluorescence.

In MCF10A treated with OA and MA, levels of ROS were decreased from 1 μM to 100 μM for OA and at 10 and 100 μM for MA (Fig 5A). MA in MCF7 increased in a dose-dependent manner ROS levels (Fig 6A). In MDA-MB-231, OA exerted an increase in ROS levels at 0,001 μM and 100 μM while MA did not alter ROS levels at any concentration tested (Fig 7A).

To induce intracellular oxidative stress, H_2O_2 was added before fluorescence measurement. Figure 5B showed a decrease of ROS levels in MCF10A for OA, however it was significant only at 1 μM. MA increased ROS levels in MCF10A in almost all concentrations (Fig 5B). For MCF7, MA appeared to increase ROS levels at the minor concentrations (Fig 6B). ROS levels in MDA-MB-231 were increased with OA from 0,01 μM to 100 μM, while MA did not show any difference statistically significant respect to control, except for 100 μM, which was decreased (Fig 7B).

OA exerted a protective action in MCF10A cells. It diminished ROS levels in basal state, and when an oxidative stress has been induced, OA continued protecting the cells, making them not such sensitive to oxidative stress. ROS can act as a trigger for carcinogenesis by permanent damage of DNA, causing mutations in p53, the tumour suppressor gene, which is frequently mutated up to 50% [23]. In this way, OA could act like an antioxidant, protecting cells in an oxidative stress microenvironment, which could lead to promote carcinogenesis [22,23]. For assessing this theory, our group studied the effects of OA and MAS in a H_2O_2-induced DNA damage.

Although MA had not this effect in MCF10A, it exerted a strong increase of oxidative stress in MCF7 in a dose-dependent manner, which continued when an oxidative stress has been induced.

This pro-oxidative effect was exerted by both compounds in MDA-MB-231. In basal state, OA appeared to have an increase of oxidative stress at minor concentrations in MDA-MB-231. And when an intracellular oxidative stress by adding H_2O_2 was induced, OA increased dramatically the oxidative stress, approximately 30% more than the control. MA showed the same effect but more slightly. So, OA had a protection role against oxidative stress in normal epithelial cells, while it had a pro-oxidant role in the highly invasive breast cancer cell line. This pro-oxidant role in breast cancer cell lines could be important, taking into account that high enough

levels of ROS may inhibit carcinogenesis by enhancing p53 expression and inducing apoptosis in the tumour cells [23].

In order to corroborate these effects in ROS levels, antioxidant catalase (CAT) enzyme activity was studied.

Determination of CAT activity

Enzymatic activity of CAT was determined by the catalase assay kit purchased from Calbiochem. With this kit, it has been measured the exactly quantity of active enzyme that each sample possess. The results are expressed like nmol/min/mL of enzyme.

It is known that ROS production is increased in cancer cells, which leads to a persistent pro-oxidative situation. Thus, antioxidant enzymes (like CAT and SOD) have a protection role in cancer cells against oxidative stress. But so many compounds are able to attack cancer cells through generation of ROS or interfering with ROS metabolism and inhibiting CAT to sensitize the cancer cells to ROS-induced apoptosis [23].

Activity of CAT measured in MCF10A were higher than the control in OA 1 and 10 μM, unless there were no statistically differences respect to control, these concentrations appeared to promote the activation of enzyme (Fig 8A). ROS levels in basal MCF10A diminished at 1 and 10 μM of OA too. So, it seemed that the decrease of ROS levels in MCF10A at these concentrations was correlated with an increase of CAT activity. Maybe, the activation of the mitochondrial antioxidant enzymes pathway was mediated by OA in MCF10A. Hence OA would act like an antioxidant for the normal epithelial cells. MA appeared to have a dose-dependent effect. CAT activity was increased as the concentrations were incremented, although it was not as marked as OA.

For MCF7, OA at 0,1 μM increased CAT production significantly, decreasing its production at higher concentrations. This result agrees with ROS levels previously described by Allouche et al. [4]. Unless 0,1 μM was not assayed in this previous work, 1 and 10 μM of OA decreased ROS levels; this correspond with the levels of CAT found in MCF7 in the present work (Fig 8B). MA did not alter the activity of CAT respect to control in MCF7 (Fig 8B) which indicates that the high levels of ROS produced by MA in MCF7 may be due to the decrease of another antioxidant enzyme different from CAT, like superoxide dismutase (SOD), or another different mechanism.

Although there were no statistically significant differences in MDA-MB-231, there was a slight decrease in the activity of CAT at OA 1 and 10 μM (Fig 8C); this could indicate that these compounds could be able to inhibit the action of this enzyme, not protecting the cell against an increase of oxidative stress.

<u>Effects in H_2O_2 – induced DNA damage</u>

To study the protection of these triterpenes against injury induced to DNA, H_2O_2 was used for promote single-strand DNA breaks. Results are expressed like the percentage of Olive_TM for each cell line. 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), so this measure gives us information about the injury induced to DNA and their capacity of self-repair [24].

Early it was explained that ROS can damage DNA in normal cells, triggering carcinogenesis. However, adequate levels of ROS may show opposite effects in cancer cells by enhancing p53 expression and inducing apoptosis in the tumour cell [23].

Taking this into account, we want to know if normal epithelial cells were able to self-repair, once DNA is damaged. And if pre-treatment with these compounds were related with this DNA repair.

Our results showed that for MCF10A, OA at 1 µM protected against H_2O_2 injury to DNA, producing less DNA breaks than the control (Fig 9A). MA possessed the same effect at 10 µM but it has to be noted that at this concentration MA was pro-apoptotic for the normal epithelial cell line MCF10A. So, this result is probably due to cells which were the percentage that remain alive in cytotoxic and proliferation assay, and were not affected by MA.

Unless at 1 µM MA does not have pro-apoptotic effects, in this assay appear to promote damage to DNA, which support results obtained in detection of ROS levels after added H_2O_2. MA could act like a pro-oxidant in these cells, increasing ROS in the first moments of treatment; that with addition of H_2O_2, resulting in the damage to DNA that showed our results. Even though, the effect at this concentration seem not be the same along the time, like the proliferation results have showed.

For MCF7, MA did not show significant differences respect to control (data not shown).

For MDA-MB-231, OA promoted an increase in Olive_TM at 10 µM, and unless it was not significant, it appeared also to increase at 0,1 µM. MA induced more injury to DNA, increasing the Olive_TM at all concentrations tested in MDA-MB-231 (Fig 9B). Consequently, for the highly invasive breast cancer cell line, both compounds only with 4 h of treatment, promoted a high damage to DNA. Therefore, the cytotoxic effects of oleanolic acid observed in MDA-MB-231 appear to be connected with the increase of ROS levels observed that in turn promote damage to DNA.

Even so, large amounts of ROS are undoubtedly able to oxidize the biological molecules such as amino acids, proteins, lipids, and DNA, leading to severe damage

and finally cell death [23], like OA showed for MDA-MB-231. For the same reason, any compound which would able to protect ROS increase and to minimize damage to DNA, could protect the cells against a pro-oxidative microenvironment, making these cells not susceptible of oxidization, effect due to OA in MCF10A. Our results showed that OA had two different effects against damage to DNA, depend on it is a non-tumorigenic breast cell line or a highly invasive breast cancer cell line.

Discussion

OA and MA are triterpenes found in virgin olive oil. Triterpenes are present in several plants, like olive tree, and consequently in virgin olive oil. Several studies indicate the antitumoral effects of triterpenes in different kinds of cancer [22], but until now, there is not scientific data about the effect that OA and MA have in human breast cancer cells. In the present work, our group focus on the study of the effects of these two natural compounds in human normal breast cells and in human breast cancer cells.

Oleanolic acid has been described recently to be pro-apoptotic in oestrogen receptor negative/progesterone receptor negative/HER2 negative (ER-/PR-/Her2-) breast cancer cells [25]; and patients with ER- genotype are considered to have a more aggressive metastatic breast cancer than patients with ER+ [26]. Chu et al. described the action of BN107 (extract with several terpenoidal saponins similar to OA) and with 70 μg/mL, this compound promotes apoptosis in MCF10A (ER-) and in MDA-MB-231 (ER-) [25]. They conclude that BN107 and OA are strong inhibitors of the Akt/mammalian target of rapamycin (mTOR) pathway, which may negate chemoresistance development in ER- breast cancer cells. Our results show that, unless MCF10A is ER-, OA was not able to cause cell death at minor concentrations than 10 μM; otherwise OA had antiproliferative effects (at concentrations minor than 10 μM) in the highly invasive MDA-MB-231 human breast cancer cell line, ER- too. According to this, the action of OA appears not to be related with ER expression; depending on the concentration used, OA is able to promote cell death in ER- cell lines (MDA-MB-231 and MCF10A) and ER+ cell line (MCF7) [4].

Our results show that MA inhibited the growth in the minimally invasive MCF7 human breast cancer cell line only at the highest concentration tested. Thus, they do not alter cell cycle or induce apoptosis at the concentration used previously by our group [4] or in this work. It has to be pointed out that for several assays, MCF7 cells were treated only with MA, because OA was assayed before by our group in the same concentrations. MA treatment in MCF7 was repeated due to the differences in purity of the compound assayed in this work (>98%) and the previous by our group (>80%) [4]; MA assayed in the present work showed differences in its effects on MCF7 that were not reported in the previous work.

That our results did not show apoptosis in MCF7 could be explained by the fact that generally, MCF7 cells undergoing cell death does not display some of the distinct morphological features typical of apoptotic cells such as shrinkage and cytoplasmic blebbing. Because MCF7 cell line has lost caspase-3 owing to a 47-base pair deletion within exon 3 of the *CASP*-3 gene [27]. This deletion results in the skipping of exon 3 during pre-mRNA splicing, thereby abrogating translation of the CASP-3 mRNA. It seems that caspase-3 is required for DNA fragmentation and some of the typical morphological changes of cells undergoing apoptosis. In the present work, MCF7 cells did show neither apoptosis nor changes in DNA fragmentation studied with comet assay, but a decrease of proliferation was observed with OA treatment [4] and MA treatment. Unless any signs of apoptosis are observed, MA may promote MCF7 death by increasing oxidative stress within the cell. CAT levels do not increase significantly with MA treatment, so the levels of oxidative stress are dramatically increased by this compound. This increase of ROS levels could contribute to apoptosis or cell death [28].

OA has been described to decrease the expression of Bcl-2, and increase Bax in melanoma B16F10 cells [29]. Maybe OA exerts its effect in MDA-MB-231 by this pathway, which is related with oxidative mechanism of the cell [22]. MA could have a JNK-p53-dependent mechanism or via the mitochondrial apoptotic way, both implicated with apoptosis in HT29 colon cancer cells [30,31] and related with ROS generation. So, the connection between ROS levels and cell death appear to be closed. As evidenced our results in the analysis of DNA damage, OA and MA promote DNA damage in MDA-MB-231. Anyway, more deeply studies about molecular mechanism of the effects of OA and MA in breast cancer cells have to be done for assure this.

Concentrations higher than 10 µM of OA and MA along the time inhibit normal breast cells proliferation and promote apoptosis, but concentrations lower than these could even improve the proliferation of these cells. Hence the concentration is a significant detail to take into account in this kind of compounds [22]. Very few articles describe the bioavailability of these triterpenes in humans after intake [32-34], however the concentration within the cells, after the metabolism of these compounds, is not described yet; nevertheless the concentration in which they are present in virgin olive oil are minor than in other types of olive oil [1].

Our results showed that OA acts like antioxidant in the non-tumorigenic breast cell line (MCF10A) *in vitro*. It decreased the basal oxidative stress of the cell, activating CAT action. Furthermore, when an oxidative stress was induced, the cells treated with OA decreased their levels of oxidative stress respect to untreated cells. The irreversible injuries of oxidative stress to DNA and proteins is usually prevented by antioxidants [23], in this line OA acts as an antioxidant for MCF10A protecting the cell against oxidative damage to DNA. Moreover, OA inhibited proliferation in MDA-MB-231, the highly invasive human breast cell line.

For these reasons, we might consider that OA has a potential chemopreventive activity in human breast cancer. At minor concentrations, OA is a natural compound that does not present toxicity against breast cells, it acts as an antioxidant in the normal breast cells and prevents oxidative DNA damage and besides, it has antiproliferative effects in breast cancer cells.

Conclusion

According to our results, OA has the potential to act as a chemopreventive in human breast cancer and also, it has the capacity to arrest proliferation of the metastatic breast cancer cells. Nevertheless, extreme caution should be applied in the extrapolation of the present in vitro results to potential clinical effects in human. Further studies are needed to confirm both the chemopreventive capacity of OA and the differential mechanism of action on normal versus breast cancer cells suggested by the present study.

Acknowledgements

Competing interests

The authors declare that they have no competing or financial interests.

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Table 1.

Percentage of cells									
	MDA-MB-231				**MCF10A**				
	SubG0/G1	G0/G1	S	G2/M	SubG0/G1	G0/G1	S	G2/M	
Control	0.84 ± 0.16	65.38 ± 1.25	16.18 ± 1.08	16.44 ± 2.05	0.39 ± 0.15	58.22 ± 2.93	15.90 ± 1.43	24.54 ± 1.51	
OA 0.1 µM	0.75 ± 0.25	**59.04 ± 2.99***	17.31 ± 1.58	**21.89 ± 2.09***	0.54 ± 0.27	56.86 ± 4.60	16.54 ± 2.83	24. 27 ± 1.18	
OA 1 µM	0.78 ± 0.25	59.73 ± 1.98	17.60 ± 1.66	20.76 ± 1.54	0. 37 ± 0.15	58.23 ± 3.63	15.68 ± 1.86	25.08 ± 1.23	
OA 10 µM	0.76 ± 0.37	61.06 ± 1.85	15.04 ± 1.46	**21.36 ± 0.91***	0.84 ± 0. 25	**71.45 ± 6.63***	11.58 ± 2.54	**15.14 ± 4.22***	
MA 0.1 µM	1.42 ± 0.49	61.88 ± 0.73	16.86 ± 2.25	19.28 ± 1.61	0.37 ± 0.13	58.77 ± 1.75	15.17 ± 0.20	24.43 ± 2.26	
MA 1 µM	0.72 ± 0.49	61.90 ± 0.52	16.66 ± 1.13	19.59 ± 0.84	0.59 ± 0.16	58.81 ± 3.82	16.58 ± 2.65	22.92 ± 1.88	
MA 10 µM	0.72 ± 0.01	62.36 ± 0.65	16.56 ± 1.12	19.75 ± 1.64	**64.68 ± 1.92***	**21.96 ± 1.82***	**8.01 ± 1.15***	**4.56 ± 1.24***	

Table 2.

Percentage of cells	MDA-MB-231			MCF10A		
	Live	Apoptotic	Death	Live	Apoptotic	Death
Control	87.64 ± 3.16	8.92 ± 2.15	1.33 ± 0.48	92.43 ± 1.43	5.92 ± 1.40	1.63 ± 0.53
OA 0.1 µM	90.66 ± 4.28	8.43 ± 4.04	0.90 ± 0.36	94.43 ± 0.71	3.57 ± 1.31	1.97 ± 0.61
OA 1 µM	86.72 ± 3.27	11.83 ± 3.28	1.43 ± 0.24	94.91 ± 0.74	2.16 ± 0.79	2.90 ± 1.00
OA 10 µM	88.22 ± 2.78	10.20 ± 3.36	1.56 ± 0.61	70.40 ± 16.09	**17.18 ± 8.22***	12.41 ± 7.89
MA 0.1 µM	90.81 ± 3.29	8.12 ± 2.65	1.05 ± 0.67	92.38 ± 2.01	6.35 ± 2.30	1.26 ± 0.33
MA 1 µM	89.43 ± 5.38	7.70 ± 3.06	2.85 ± 2.33	92.35 ± 1.30	5.80 ± 1.84	1.83 ± 0.63
MA 10 µM	88.86 ± 2.41	10.13 ± 2.16	0.98 ± 0.30	5.64 ± 2.31	**78.17 ± 8.92***	16.17 ± 7.01

Figure legends

Figure 1. Effects of OA and MA in cytotoxicity. Cytotoxicity of OA and MA from 0,001µM to 100 µM in MCF10A (A), MCF7 (B) and MDA-MB-231 (C) at 24 h. Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 2. Effects of OA and MA in MCF10A proliferation. Percentage of cell proliferation in MCF10A after treatments from 0,01 µM to 100 µM of OA and MA at 24 (A), 48 (B) and 72 h (C). Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 3. Effects of MA in MCF7 proliferation. Percentage of cell proliferation in MCF7 after treatments from 0,01 µM to 100 µM of MA at 24 (A), 48 (B) and 72 h (C). Statistically differences are represented by (†) for MA at p< 0,05 respect to untreated control.

Figure 4. Effects of OA and MA in MDA-MB-231 proliferation. Percentage of cell proliferation in MDA-MB-231 after treatments from 0,01 µM to 100 µM of OA and MA at 24 (A), 48 (B) and 72 h (C). Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 5. ROS produced by OA and MA. ROS levels present in MCF10A in basal state (A) and with H_2O_2 burst (B), after treatments with OA and MA from 0,0001 µM to 100

μM. Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 6. ROS produced by OA and MA. ROS levels present in MCF7 in basal state (A) and with H_2O_2 burst (B), after treatment with MA from 0,0001 μM to 100 μM. Statistically differences are represented by (†) for MA at p< 0,05 respect to untreated control.

Figure 7. ROS produced by OA and MA. ROS levels present in MDA-MB-231 in basal state (A) and with H_2O_2 burst (B), after treatments with OA and MA from 0,0001 μM to 100 μM. Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 8. Effects of OA and MA in catalase activity. CAT activity in MCF10A cells (A), MCF 7 cells (B) and MDA-MB-231 cells (C) treated with OA and MA at 0,1 μM, 1 μM and 10 μM. Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to untreated control.

Figure 9. Effects of OA and MA in induced oxidative damage to DNA. OLIVE_TM represented in MCF10A cells (A) and MDA-MB-231 cells (B) treated with OA and MA at 0,1 μM, 1 μM and 10 μM. Statistically differences are represented by (*) for OA and (†) for MA at p< 0,05 respect to H_2O_2 control.

Table 1. Effects of OA and MA in cell cycle. Distribution of cell cycle of MDA-MB-231 and MCF10A treated with OA and MA at 0,1 μM, 1 μM and 10 μM at 24 h. Result expressed as percentage of cells ± SEM. Statistically differences are represented by (*) at p< 0,05 respect to untreated control.

Table 2. Effects of OA and MA in apoptosis. Apoptosis of MDA-MB-231 and MCF10A cells treated with OA and MAS at 0,1 μM, 1 μM and 10 μM at 24 h. Result expressed as percentage of cells ± SEM. Statistically differences are represented by (*) at p< 0,05 respect to untreated control.
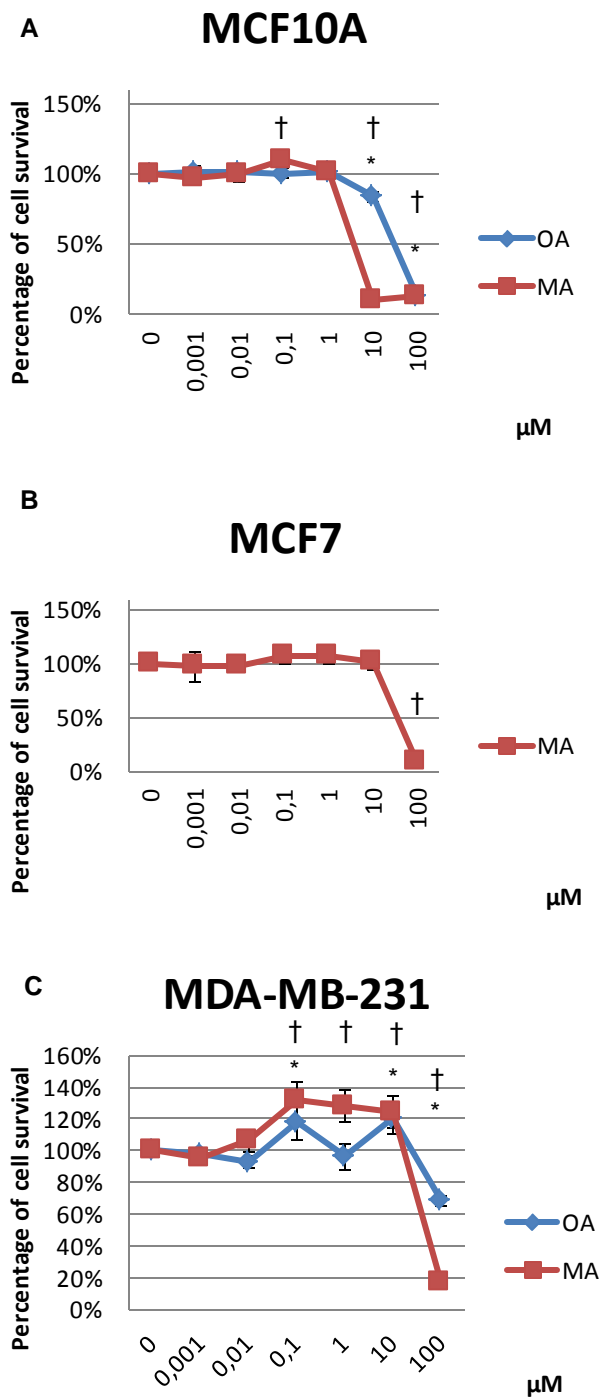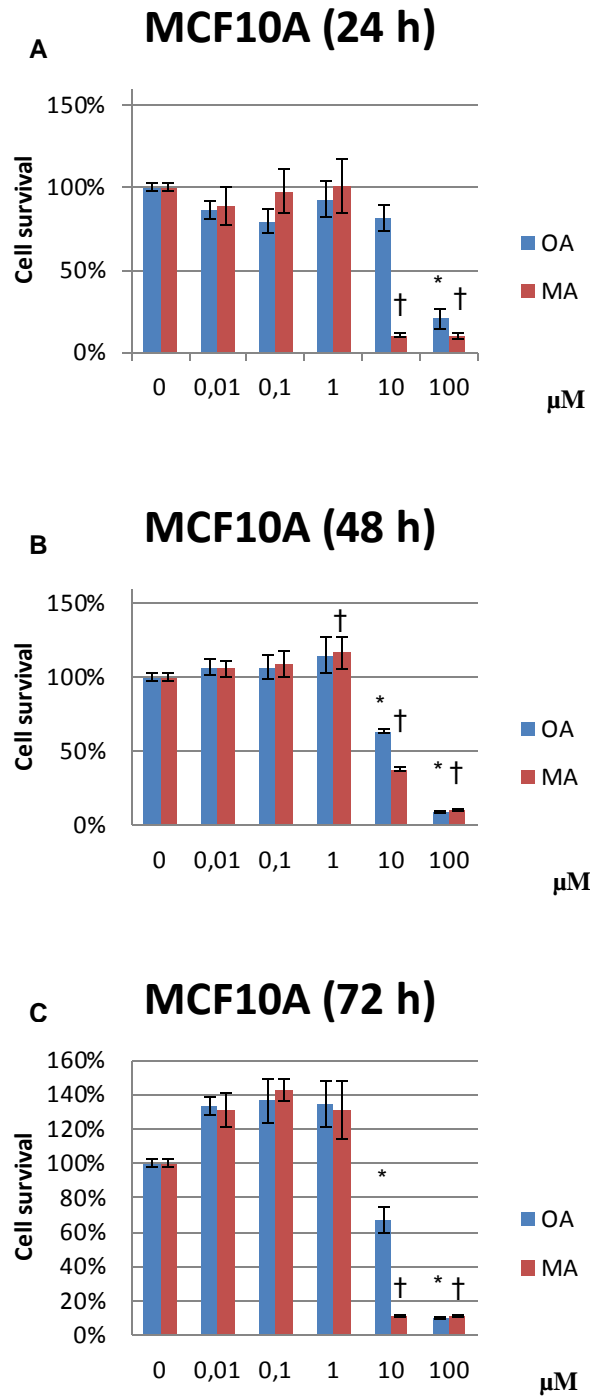
A **MCF10A**

B **MCF7**

C **MDA-MB-231**

Figure 1.

MCF10A (24 h)

A

MCF10A (48 h)

B

MCF10A (72 h)

C

Figure 2

A

MCF7 (24 h)

B

MCF7 (48 h)

C

MCF7 (72 h)

Figure 3

A MDA-MB-231 (24 h)

B MDA-MB-231 (48 h)

C MDA-MB-231 (72 h)

Figure 4

A

MCF10A Basal

B

MCF10A H$_2$O$_2$

Figure 5.

A MCF7 Basal

B MCF7 H₂O₂
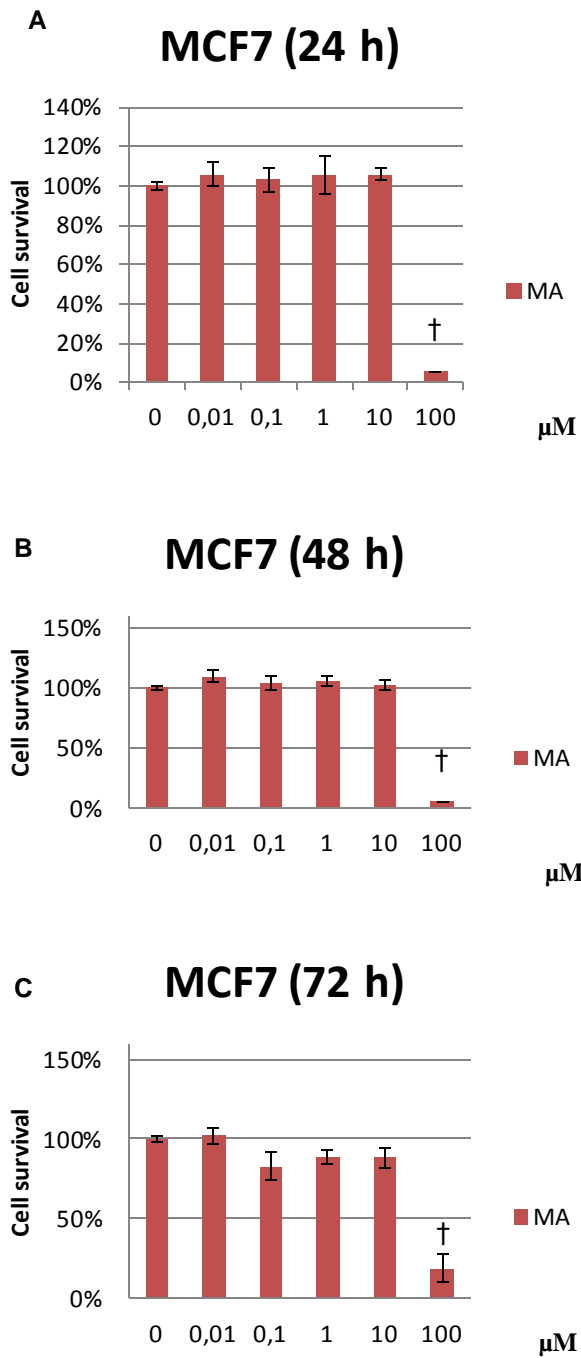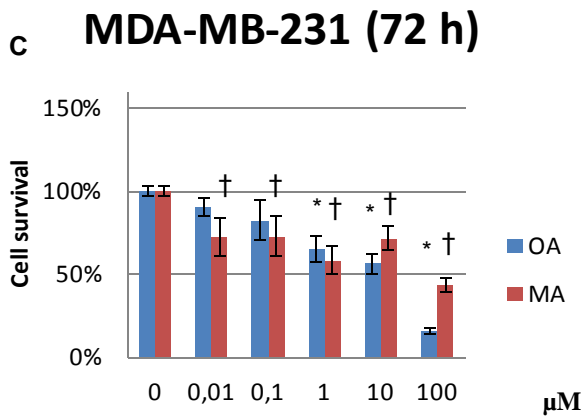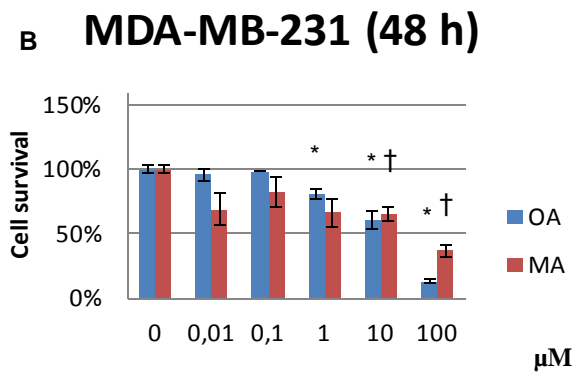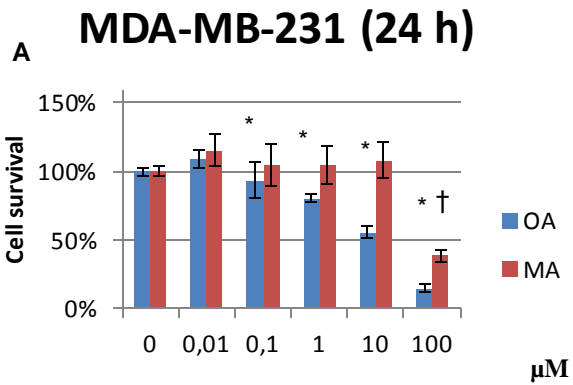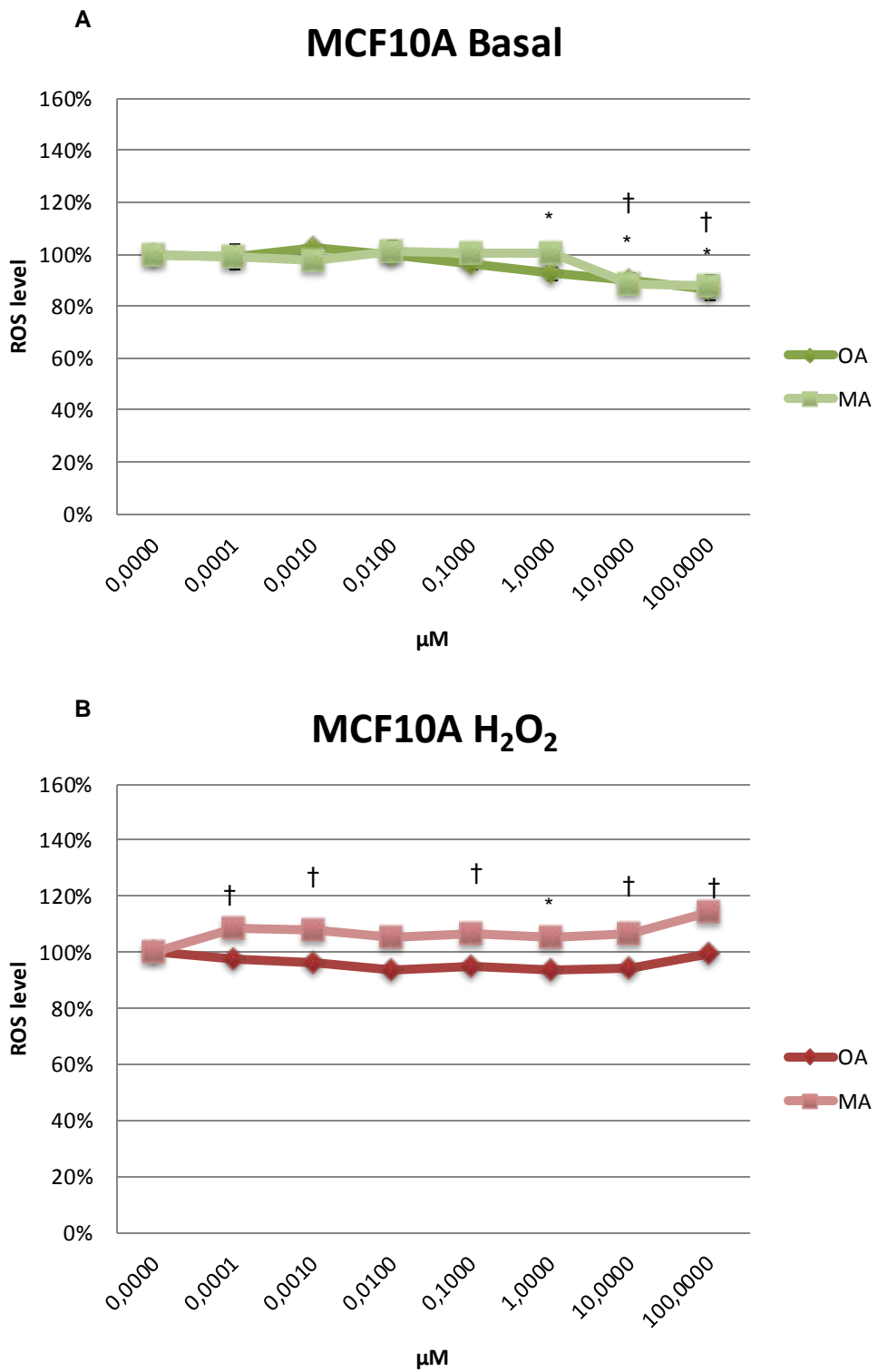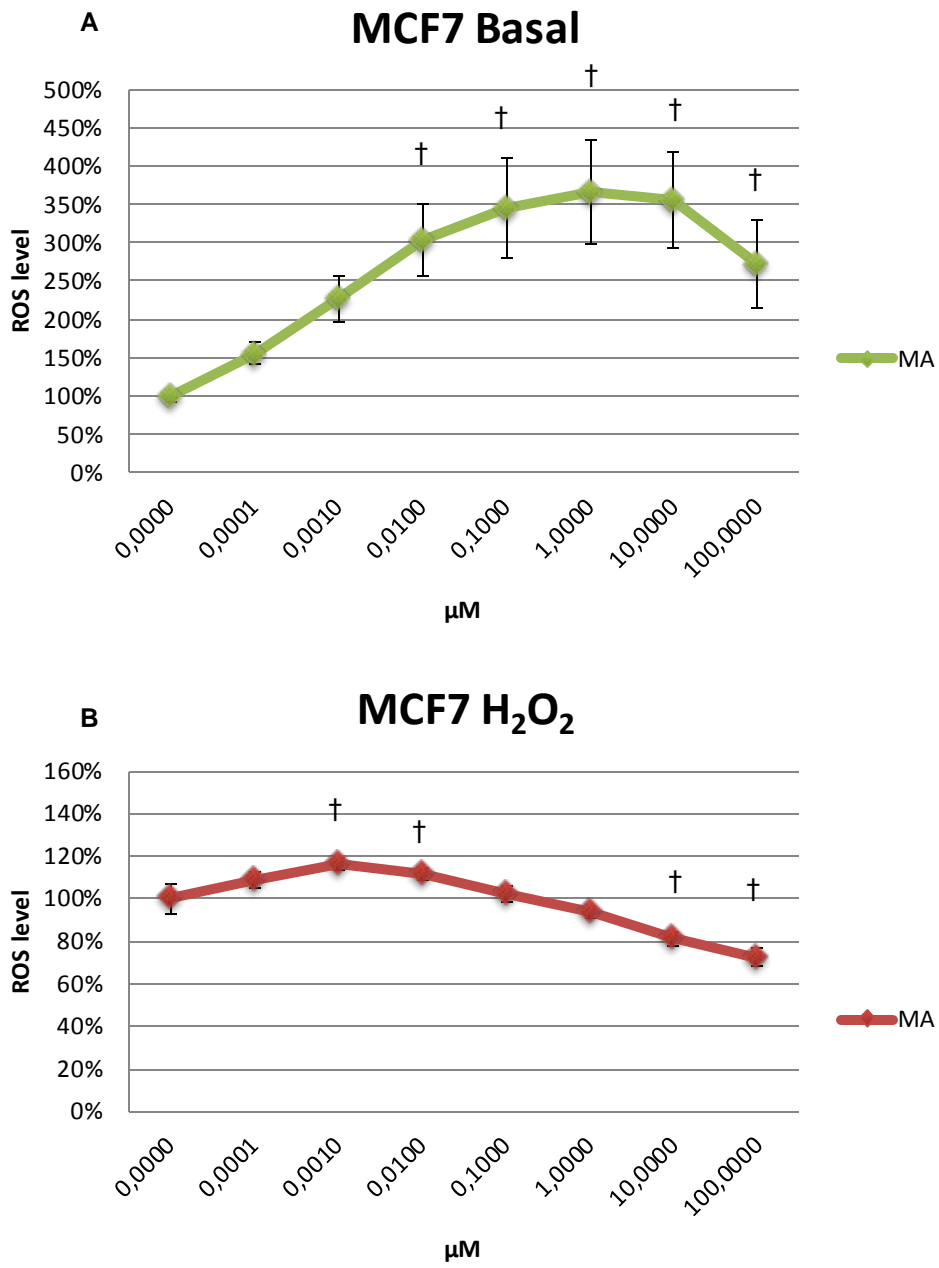
Figure 6.

MDA-MB-231 Basal
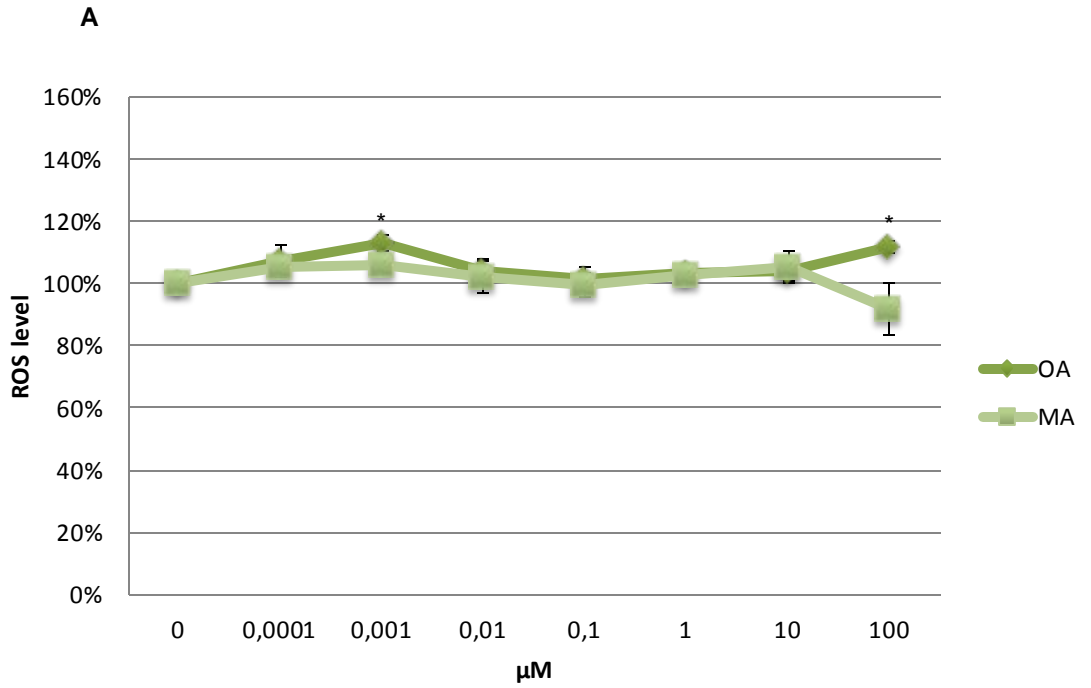
MDA-MB-231 H$_2$O$_2$

Figure 7.

Figure 8.

A

MCF10A

B

MDA-MB-231
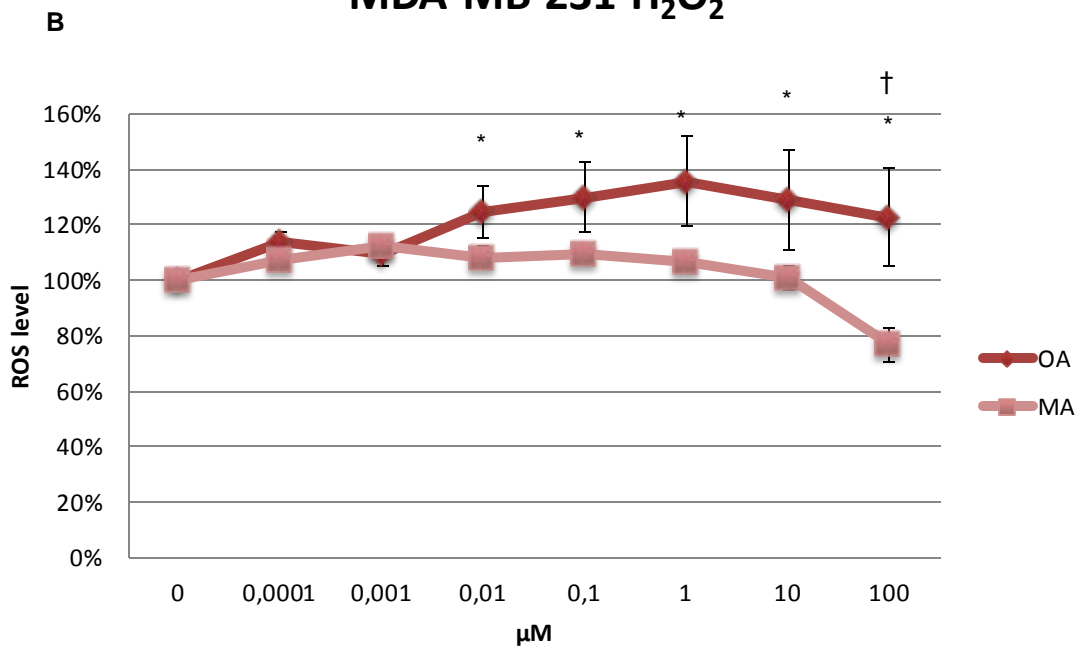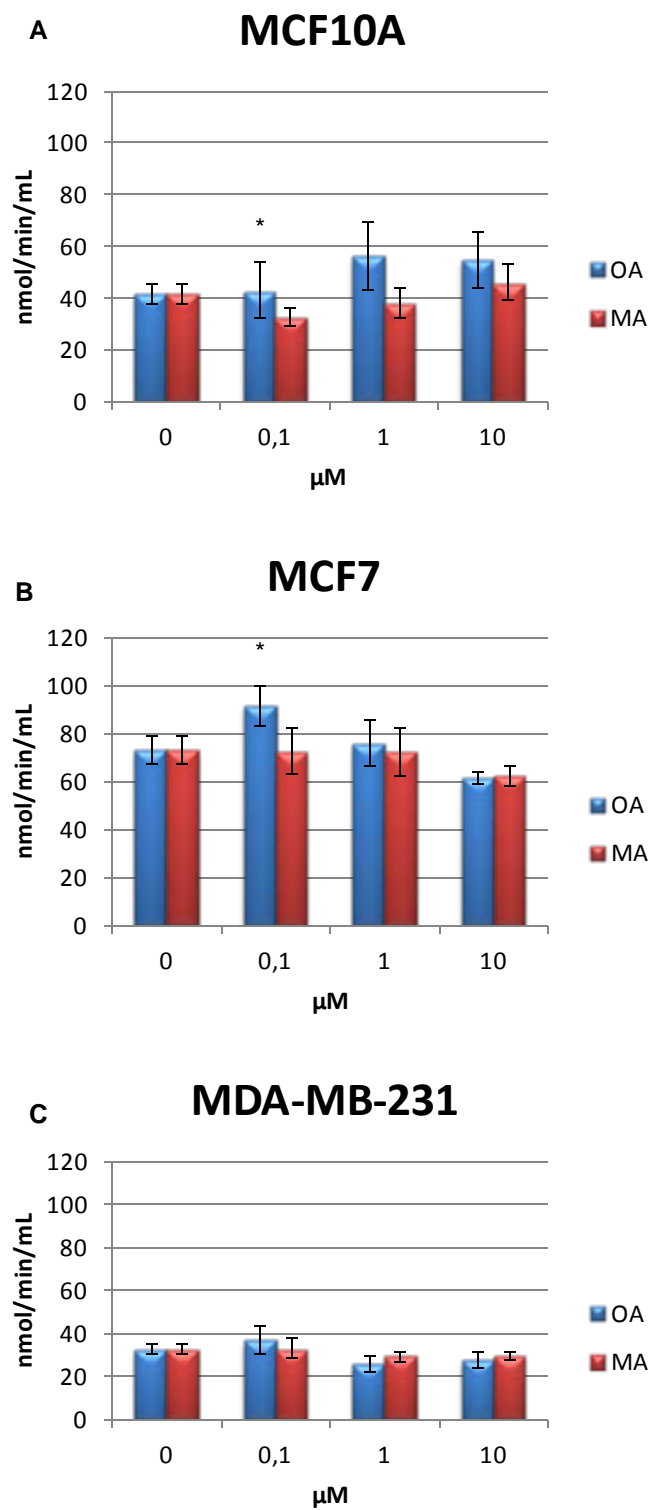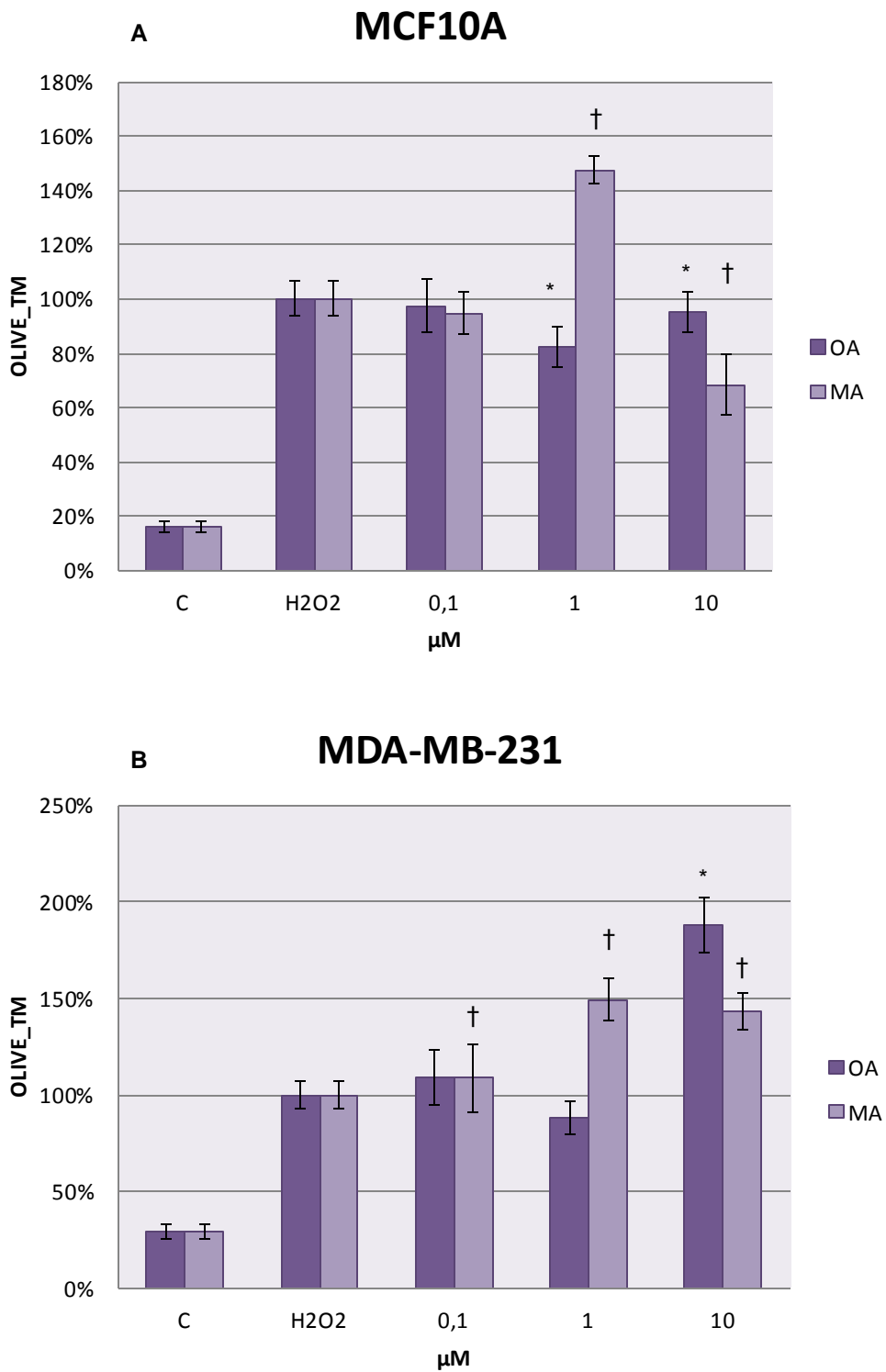
Figure 9.

Trabajo experimental y resumen global de los resultados

2. **The differential localization of a methyl group confers to two triterpenes present in the olives a different anti-breast cancer activity.**

Trabajo enviado a la revista "Food & Function".

- Índice de Impacto: 2.907
- Categoría perteneciente: Ciencia de la alimentación y la tecnología
- Cuartil en la categoría: Q1
- Rango en la categoría (2013): 16/123
- Fecha de envío: 2-09-2014

The differential localization of a methyl group confers to two triterpenes present in the olives a different anti-breast cancer activity.

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The antitumor activity depends on the location of CH₃ group.

Abstract

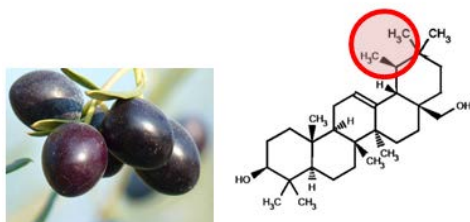Uvaol (UV) and erythrodiol (ER) are two triterpenic dialcohols present in the minor fraction of virgin olive oil, in leaves and in the pomace of olives. These triterpenes possess the same chemical structure, only differs in the location of a methyl group. It has been reported that they have antitumoral effects in leukemic cells, in skin mice tumours and, finally, in astrocytoma cells, but there are no evidences about their effect in highly invasive human breast cancer cells and human epithelial breast cells. For this purpose, we have evaluated their cytotoxic activities as well as, their effects on cell proliferation, cell cycle profile, apoptotic induction, oxidative stress and DNA oxidative damage in both, highly invasive human breast cancer cells (MDA-MB-231) and human epithelial breast cells (MCF10A). Our results showed that the change in the location of a methyl group, which characterized to these two triterpenes, is responsible for the different effects that both compounds exert on both, highly invasive breast cancer cells and epithelial breast cells. UV protected from damage to DNA in both cell lines, while ER enhances damage to DNA in them. Thus, ER promoted apoptosis and arrest cell cycle in human epithelial breast cells. So, both compounds

differ in their action in human breast cells apparently by the different location of only a methyl group.

Introduction

Uvaol (UV) and erythrodiol (ER) are two compounds present in leaves and fruit of olive tree and for instance in virgin olive oil [8]. These two compounds possess two hydroxyl groups in remote positions, and differ in a methyl group location (Fig 1). UV and ER are described to possess multiple activities against different kinds of cancer cells [9-12]. But until now, there is no reference about the role that these two dialcohols possess in metastatic breast cancer cells or in human non-tumorigenic breast cells.

On the other hand, it is worldwide known the healthy benefits of virgin olive oils and the multiple qualities that it possesses in the prevention of several diseases [1-7], among them breast cancer. It is known that in carcinogenesis ROS levels show altered, in fact, ROS production is increased in many cancer cells (*in vitro* and *in vivo)* which results in a persistent pro-oxidative situation and creation of more H_2O_2 than normal cells. Controlling ROS levels inside cancer cell we could activate apoptosis pathway in the cell, or even prevent carcinogenesis in breast normal cells. For this reason, some antioxidants possess anticancer activity and are beneficial in cancer chemoprevention due to, for example, the regulation of Akt-ROS (protein kinase B-ROS) pathways [14], which are implicated in intracellular ROS production. UV and ER possess antioxidant activity against lipid peroxidation in rats [15], so they could be a good option for controlling ROS levels inside the cells.

Taking into account that control of ROS could play a role in the prevention of breast cancer, it is interesting to know if UV and ER, two triterpenes with antioxidant activity, have the capacity to prevent breast cancer development, or even if they have antitumor properties. For this reason, the purpose of this study was to examine the effects of UV and ER in human epithelial breast cells (MCF10A cells) and in highly metastatic breast cancer cells (MDA-MB-231 cells).

Results

Cytotoxicity

In the MCF10A cell line (Fig 2A), our results showed a decrease of survival at 10 and 100 μM for both compounds. But in the MDA-MB-231 cell line (Fig 2B) we observed a decrease of survival only at 100 μM for both compounds; in fact both compounds were capable of increase the number of cells at minor concentrations than 100 μM.

Proliferation

UV did not alter MCF10A proliferation, except for the concentration of 100 μM. At this concentration, proliferation after 48 h and 72 h of treatment was affected and a

statistically significant decrease of cell proliferation was observed (Fig 3). ER was antiproliferative only at 100 µM and after 72 h of treatment (Fig 3).

In MDA-MB-231, proliferation was affected only for UV at 100 µM at the three time-points assayed. ER was antiproliferative only at 100 µM after 72 h of treatment (Fig 4).

<u>Cell Cycle</u>

UV did not show any statistical difference neither in MCF10A (Fig 5) nor in MDA-MB-231 (data not shown). However, ER at 10 µM arrested cell cycle in SubG0/G1 and in G0/G1 phases in MCF10A, while in MDA-MB-231 no difference was observed respect to the control (data not shown).

<u>Apoptosis Analysis</u>

Neither UV nor ER promoted apoptosis in MDA-MB-231. ER at 10 µM promoted statistically significant apoptosis in MCF10A (Table 1).

<u>Analysis of intracellular reactive oxygen species</u>

UV and ER in MCF10A cells promoted an antioxidant effect, decreasing ROS levels respect to untreated control (Fig 6A).

After inducing intracellular oxidative stress by addition of H_2O_2, ROS levels decreased respect to the control when cells were previously treated with UV or ER at all concentration assayed (Fig 6B).

In MDA-MB-231, ROS levels decreased in cells treated with UV and ER. In fact, with UV at 10 µM we observed the maximum decrease of ROS levels (73%) respect to the control (100%) (Fig 7A).

When H_2O_2 was added, ROS levels decreased at 10 and 100 µM of UV and ER treatments, but not at minor concentrations (Fig 7B).

<u>Catalase activity</u>

There were not statistically significant differences in catalase activity after treatments with UV and ER (data not shown).

<u>Analysis of Comet Assay</u>

In MCF10A cells UV at 0,1 µM reduced the damage to DNA in more than 50 % respect to the control. But this effect was lost at 1 µM where the damage to DNA was increased. ER at 10 µM dramatically promoted DNA damage in MCF10A cells with a percentage about 200 % (Fig 8A). In order to evaluate UV and ER ability to protect against oxidative DNA damage, cells were exposed to H_2O_2. When an oxidative

damage is induced to cells, UV and ER increased the damage to DNA respect to control in MCF10A (Fig 9A).

In MDA-MB-231 cells UV at 0,1 and 1 µM decreased damage to DNA with respect to control. Otherwise, ER at 10 µM promoted damage to DNA (Fig 8B). When cells were exposed to H_2O_2, UV at 0,1 µM and ER at 1 and 10 µM promoted damage to DNA in breast cancer cells (Fig 9B).

Discussion

UV and ER are present in leaves, olives and virgin olive oil from *Olea europaea* [16]. Both compounds have identical chemical structure except for a different location of a methyl group. These compounds have been described to possess different effects related to control reactive oxygen species (ROS) [9, 17-19]. Our results showed that ROS levels were diminished respect to the control in both human breast cell lines (MCF10A and MDA-MB-231) after treatment with UV and ER. So, both compounds are antioxidant in these cells such as in MCF7 [9]. But when an oxidative stress was induced by H_2O_2, concentrations ranged between 0,0001 and 1 µM did not exert antioxidant effect in MDA-MB-231, while in MCF10A they still exert antioxidant effect. Thus, it seems that both compounds interfere with ROS levels inside the cells.

It is known that ROS are implicated in the redox regulation of the cell function. The role of oxygenated radicals has been highly cleared in regulation of gene expression, cell oxidative injuries and cytotoxic activity of immune system. In cancer, the balance of ROS within the cancer cell could derivate in promote apoptosis and necrosis of the malignant cells or could act as a trigger for carcinogenesis by permanent damage of DNA, causing mutations in p53, the tumour suppressor gene, which is frequently mutated up to 50% [13].

Under normal metabolic conditions about 2-5% O_2 consumed by mitochondria is converted to ROS; ROS include radical species such as superoxide anion, hydrogen peroxide, highly reactive hydroxyl radical etc... Aerobic organisms protect themselves from ROS using endogenous enzymes (catalase, superoxide dismutase, glutathione peroxidase) and by dietary antioxidants that accumulate within the cells (ascorbic acid, α-tocopherol...). But when an increase of ROS is persistent (in carcinogenesis for example), it can affect to DNA, proteins or lipids [20]. The results obtained in this work show that, when the normal epithelial cell line is in the basal state, UV at 0,1 µM protects them from oxidative damage. In MDA-MB-231 this compound had the same effect, it prevented from oxidative damage but interestingly ER had the contrary effect in both cell lines tested (MCF10A and MDA-MB-231).

ROS can interact with DNA and promote damage of DNA in cells which can take many forms (specifically oxidized purine and pyrimidine bases, strand breaks, sister

chromatid exchanges, formation of micronuclei ...). For assess the effects of ROS on DNA in both cell lines after treatment with UV and ER, the comet assay was made. In both cell lines tested, UV acted as antioxidant protecting the cells against DNA damage (according with the results obtained in oxidative stress measurement), while ER promoted damage to DNA. But when cells were exposed to oxidative damage (H_2O_2 was added), UV and ER at minor concentration enhanced the damage to DNA in both breast cell lines, changing their behaviour when we altered the intracellular ROS levels.

Both compounds at high concentrations promoted cell death, but our results only showed apoptosis in MCF10A with ER at 10 μM. This compound at 10 μM was able to increase DNA damage in basal and in stress conditions induced by H_2O_2. ER could promote stress, and stress could activate c-Jun N-terminal kinase (JNK) pathway, that is known to be involved in apoptosis regulation in several cellular types [19]. In the present study, cells after 4h of treatment with ER at 10 μM promoted DNA damage which could derivate in apoptosis, also observed by Martín *et al* [18] in an astrocytoma cell line, 1321N1. This accumulation of ROS is also described in bladder cancer cells (NTUB1) where ER was capable only with 5 and 10 μM to increase ROS production and arrest the cell cycle in G0/G1, with the correspond apoptotic cell death in 24h [18]. So, it appears that ER could enhance DNA damage due to reactions with intracellular ROS levels. Thus, ER promoted apoptosis in the normal epithelial cell line while UV was not; and it should be pointed out that the only difference between these two compounds is the CH_3 group position.

The different roles that UV and ER had in breast cells appear to be related with two factors, the "origin" of cells and the position of CH_3 group. Above is described the different effect (once an oxidative stress was induced) in ROS levels within the cells, so the effect was different if it was a cancer cell or not. And in the same concentration, UV protected against DNA damage in MCF10A and MDA-MB-231, while ER promoted the opposite effect, enhancing DNA damage in both cell lines. So both compounds act in oxidative stress inside the cell, but with differences due to their chemical structure.

Experimentals

Chemicals

Uvaol (UV) CAS [545-46-0] (purity ≥98,5%) and erythrodiol (ER) CAS [545-48-2] (purity ≥97%)was purchased from Extrasynthese (Genay, FRANCE). The following were purchased from Sigma-Aldrich Co. (St Louis, MO, USA): Hepes solution; Sodium Pyruvate solution; Non-Essential Amino Acids mixture 100× (NEAA); 2,7-dichlorofluorescin diacetate (DCFH-DA) CAS [4091-99-0] (purity ≥97%); 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%); phosphate buffered saline (PBS); (S)-(+)-

camptothecin (CPT) CAS [7689-03-4] (purity ≥90%) and Triton X-100. Foetal Bovine Serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, AUSTRIA). TrypLE Express, HuMEC ready medium, Minimum essential medium with Eagle's salts (MEM) and Phenol-Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from Gibco® Life Technologies Ltd (Paisley, UK). Methanol dry (max 0,005%) and ethanol absolute PRS was purchased from Panreac Quimica S.L.U. (Barcelona, SPAIN). CellTiter-Blue® Cell Viability Assay was obtained from Promega Corporation (Madison, WI, USA). Phosphate buffered saline (1X, Dulbecco's) (PBS) was purchased from Applichem GmbH (Gatersleben, GERMANY). Culture plates were obtained from Starlab (Hamburg, GERMANY). The PI/RNase Staining Buffer kit was obtained from BD Biosciences Pharmigen (San Diego, CA, USA). Annexin-V FITC kit was purchased by Miltenyi Biotec (Cologne, GERMANY). The Comet Assay kit was obtained from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD, USA). The Catalase Assay kit was purchased by Merck KGAA (Darmstadt, GERMANY).

Cell culture and treatments

Highly invasive MDA-MB-231 human breast cancer cells (oestrogen and progesterone receptor-negative) and immortalized non-tumorigenic MCF10A human breast epithelial cells (oestrogen receptor-negative), were obtained from American Type Culture Collection (ATCC, Manassas,VA, USA). Breast cancer cells (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 grown in HuMEC Ready Medium. All cell lines were maintained at 37 ^{o}C in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments.

Except for the assays which specified the opposite, cells were treated with 0,1 µM, 1 µM and 10 µM of uvaol (UV) and erythrodiol (ER) for 4 h.

Cytotoxicity Assay

Cell survival, measured as the cellular growth of treated cells *versus* untreated controls, was carried out in MCF10A and MDA-MB-231 using an XTT-based assay according to Scudiero *et al.* [22] with some modifications. Briefly, cells were seeded into 96-well culture plates in a total volume of 100 µL per well (5 x 10^3 cells/well for MDA-MB-231 and 2,5 x 10^3 cells/well for MCF10A). After overnight incubation to allow cell attachment, 100 µL of fresh medium was added containing increasing concentrations from 0,001 µM to 100 µM of UV and ER for 24 h. Thereafter, cells were incubated with XTT in Phenol-Red free RPMI medium for 3 h at 37 ^{o}C with 5% CO_2, and absorbance was measured at 450nm wavelength (620nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

% viable cells = [(A$_{treated\ cells}$) / (A$_{control}$)] x 100

Where *A* is the difference in absorbance between optical density units (*A = OD$_{450}$ – OD$_{620}$*). All measurements were performed in quadruplicate and each experiment was repeated at least three times. As a vehicle control, cells were treated with Et-OH at the highest concentration of UV and ER used.

Cell proliferation assay

Cell proliferation, measured as the cellular growth of treated cells versus untreated controls, was carried out using CellTiter-Blue Cell Viability Assay. Briefly, cells were seeded into 96-well culture plates at 1 x 10^3 cells/well for MDA-MB-231 and 0,5 x 10^3 cells/well for MCF10A. After overnight incubation to allow cell attachment, medium was removed and replaced with fresh medium containing UV and ER from 0,01 μM to 100 μM. Plates were incubated for 24, 48 or 72 h followed by a 72 h, 48 h and 24 h proliferation period (incubation with fresh medium without UV or ER) respectively. At these time points, plates were incubated with CellTiter-Blue Cell Viability for 3 h at 37 °C with 5% CO$_2$ and the relative fluorescence units were measured in a plate reader (TECAN GENios Plus) (Ex. λ$_{485}$/Em. λ$_{595}$, Gain 60). Viability was calculated using the formula:

% viable cells = [(A$_{treated\ cells}$) / (A$_{control}$)] x 100

Where *A* are the relative fluorescence units for each sample. All measurements were performed in triplicate and each experiment was repeated at least three times. As a vehicle control, cells were treated with Et-OH at the highest concentration of UV and ER used.

Cell cycle assay

Cells were seeded in 12-well culture plates (1 x 10^5 cells/well for MDA-MB-231 and 0,5 x 10^5 cells/well for MCF10A) and incubated overnight to allow cells attachment. Then, cells were treated with 0,1 μM, 1 μM and 10 μM of UV and ER for 24 h; cells were harvested with TrypLE Express and washed with 1x PBS (Ca2+/Mg2+ free) (300 *x*g 10 min at 4 °C). After, cells were fixed with cold 70% ethanol and stored at -20 °C for at least 24 h. Subsequent to propidium iodide labelling (PI/ RNase Staining Buffer), cells were analysed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). The FlowJo program (v5.7.2) was used to calculate the percentage of cells in G0/G1, S and G2/M phases. Each 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). Briefly, cells were seeded in 12-well culture plates (1 x 10^5 cells/well for MDA-MB-231 and 0,5 x 10^5 cells/well for

MCF10A) and incubated overnight to allow cell attachment. After cells exposure to UV and ER for 24 h at 0,1 µM, 1 µM and 10 µM, cells were harvested with TrypLE Express, washed twice in cold 1x PBS (Ca2+/Mg2+ free) (300 xg 10 min at 4 ºC) and resuspended in 100 µL of Annexin Binding Buffer. Cells were stained with 5 µL Annexin V-FITC and 2 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.

Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured after 4 h of treatment with UV and ER at a range from 0,0001 µM to 100 µM, using the cell-permeable fluorescent probe, 2,7-dichlorofluorescin diacetate (DCFH-DA), as previously described by Warleta *et al.* [2] with some modifications. Briefly, cells were seeded on 96-well plate (5 x 10^3 cells/well for MDA-MB-231 and 2,5 x 10^3 cells/well for MCF10A) and after incubation with treatments, DCFH-DA (100 µM) was added for 30 min at 37 ºC with 5% CO_2. Cells were then 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:

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

Where $F_{t=0}$ is the fluorescence at $t = 0$ min and $F_{t=30}$ the fluorescence at $t = 30$ min. It has been described that the addition of H_2O_2 increases oxidative stress in cultured cells and directly damage DNA [22]. To evaluate the protective capacity of UV and ER 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).

Determination of catalase (CAT) activity

Cells were seeded into a 6-well plate at 0,5 x 10^6 cells/mL for MCF10A and MDA-MB-231. Cells were incubated overnight for cells attachment. After that, medium was changed by fresh medium with UV and ER. The assay was made according to manufacturer's protocol, for determination of the enzymatic activity of catalase.

Alkaline single-cell gel electrophoresis (Comet Assay)

Cells were seeded into 12-well plate (1 x 10^5 cells/well for MDA-MB-231 and 0,5 x 10^5 cells/well for MCF10A) and incubated overnight for cells attachment. Then cells were treated with UV and ER. After, cells were scraped and washed twice (300 xg 10 min, 4 ºC) with cold 1X PBS (Ca2+/Mg2+ free). They were resuspended in 1 mL of

cold 1x PBS. In order to evaluate UV and ER ability to protect against oxidative DNA damage, cells were exposed for 10 min to 50 μM H_2O_2 at 4 ^{o}C. After that, the comet assay was performed according to Warleta *et al* [2].

<u>Slide scoring and analysis</u>

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). Twenty five cell images were randomly characterized per sample using 20x magnifications. 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.

Olive_TM = [(tail (mean) – head (mean)) x tail (% DNA)] / 100

<u>Statistical analysis</u>

Results are displayed as the mean of at least three independent experiments (±SEM), and results are expressed as a percentage relative to the untreated control, which was set 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.

Conclusion

We could assume that both compounds differs in their actions in both type of cells tested (human epithelial breast cells and highly invasive breast cancer cells), which may be due to the difference in the methyl group location of their structure. UV could be able to prevent damage to DNA in breast normal epithelial cells while ER could act increasing oxidative damage in breast cancer cells. Nevertheless, more studies are needed for assure these effects in both normal epithelial breast cells and breast cancer cells.

Acknowledgements

Competing interests

The authors declare that they have no competing or financial interests.

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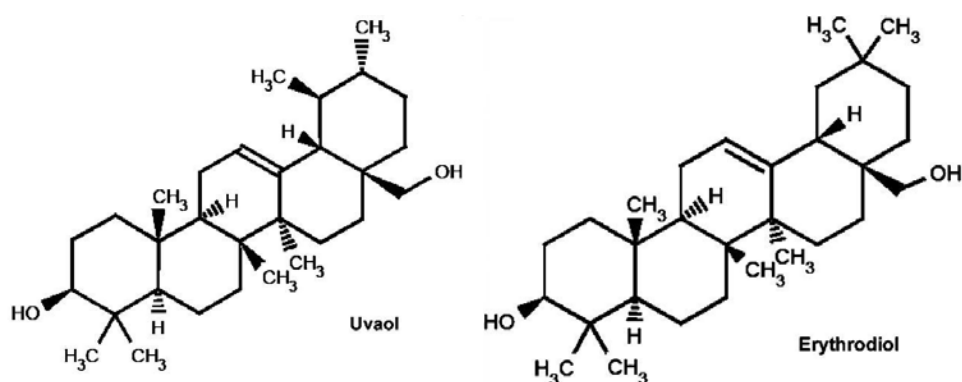
Figure 1. Chemical structure of uvaol and erythrodiol.
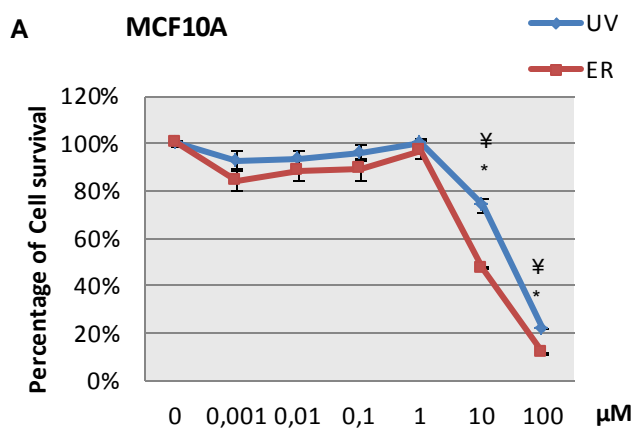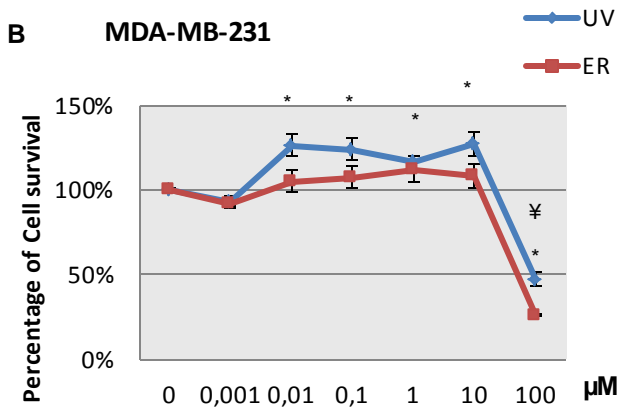
Figure 2. Cytotoxicity of UV and ER after treatment in a range between 0,001 μM to 100 μM in MCF10A cells (A) and MDA-MB-231 cells (B). Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences at p<0,05 for UV and ER are represented by (*) and (¥) respectively.
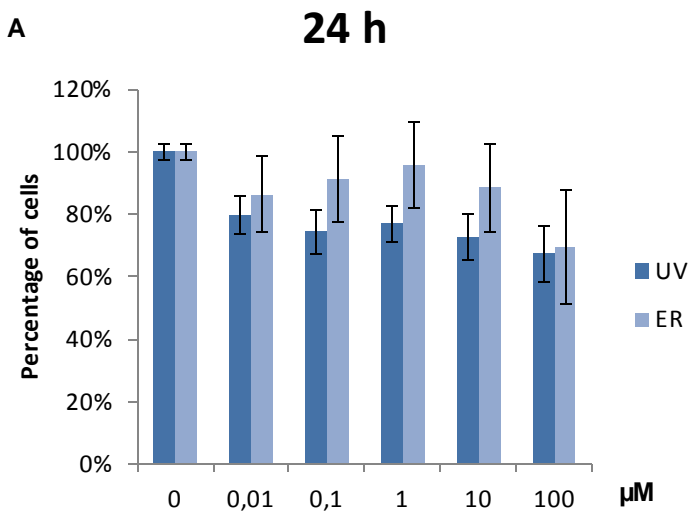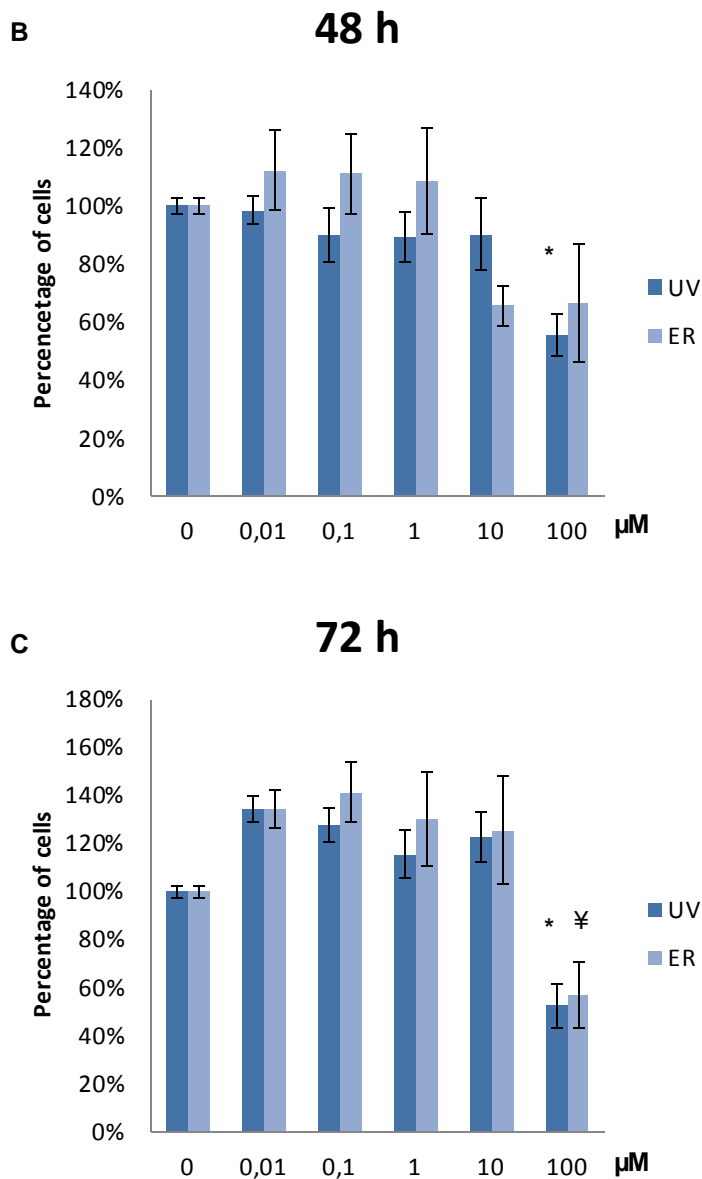
Figure 3. Proliferation of MCF10A cells after treatments with UV and ER in a range of concentrations from 0,01 μM to 100 μM at 24h (A), 48h (B) and 72h (C). Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.

A

24 h
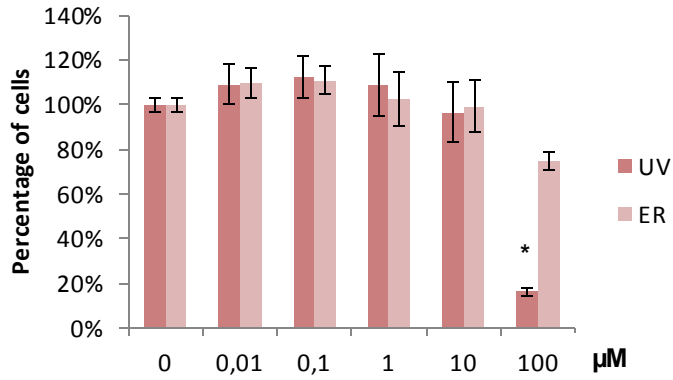
B

48 h
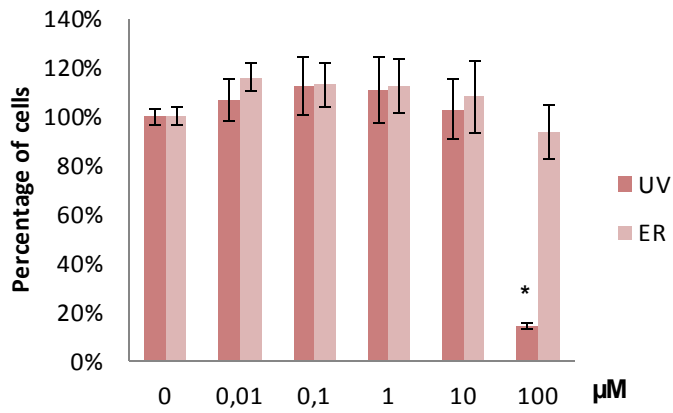
C

72 h
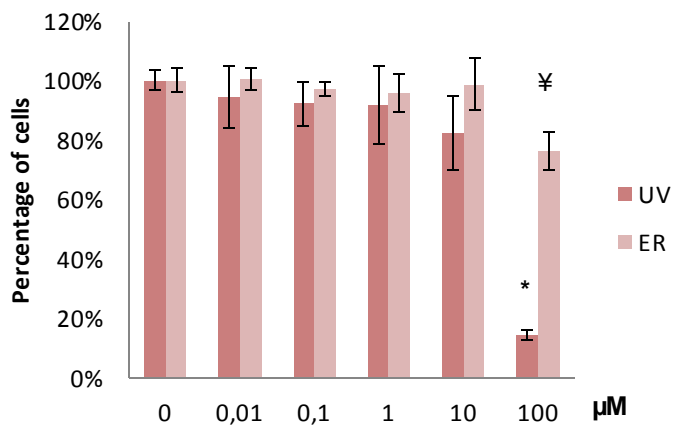
Figure 4. Proliferation of MDA-MB-231 cells after treatments with UV and ER in a range of concentration of 0,01 μM to 100 μM at 24h (A), 48h (B) and 72h (C). Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.
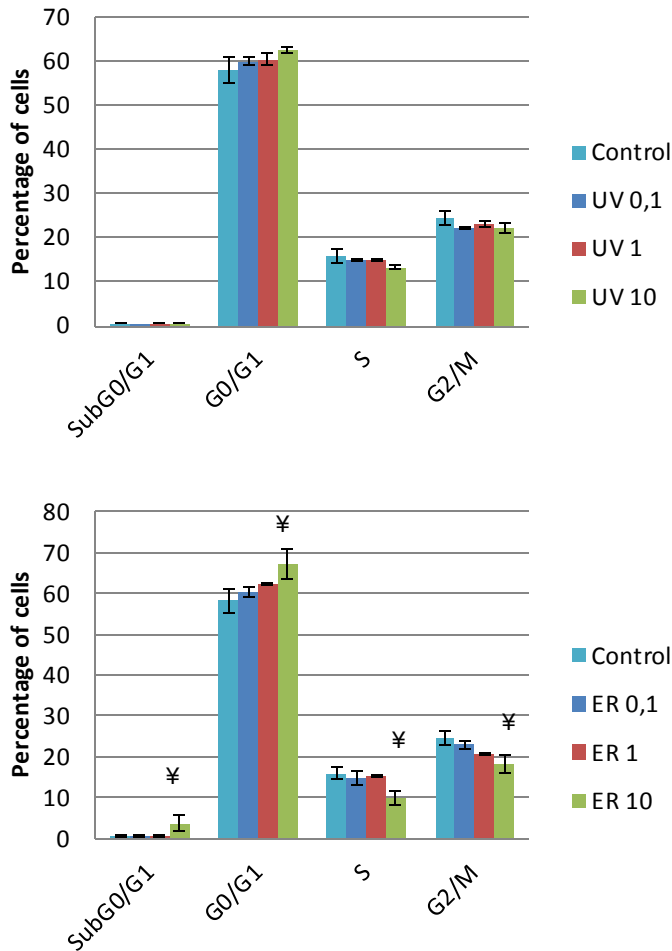
Figure 5. Cell cycle represented as percentage of cells in the different cell cycle phases of MCF10A cells after treatments with UV and ER at 0,1 μM, 1 μM and 10 μM. Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.

	MCF10A			MDA-MB-231		
	Live	Apoptotic	Death	Live	Apoptotic	Death
Control	92,43 ± 1,42	5,92 ± 1,40	1,63 ± 0,53	87,64 ± 3,16	8,92 ± 2,15	1,33 ± 0,48
UV 0,1 µM	89,64 ± 2,40	7,31 ± 2,04	3,04 ± 1,97	85,53 ± 6,13	11,94 ± 4,06	2,51 ± 2,07
UV 1 µM	89,50 ± 3,99	9,05 ± 4,49	1,44 ± 0,56	85,53 ± 6,59	11,56 ± 4,28	2,88 ± 2,31
UV 10 µM	82,40 ± 6,20	14,44 ± 7,67	3,14 ± 1,53	85,46 ± 3,90	11,83 ± 1,92	2,68 ± 2,01
ER 0,1 µM	88,97 ± 4,73	9,68 ± 5,30	1,34 ± 0,57	84,61 ± 6,62	13,08 ± 4,97	2,29 ± 1,67
ER 1 µM	88,94 ± 3,86	8,86 ± 4,62	2,17 ± 0,78	84,40 ± 4,61	13,08 ± 2,83	2,50 ± 1,78
ER 10 µM	65,85 ± 15,21	**27,63 ± 18,31¥**	6,51 ± 4,02	89,68 ± 1,07	7,73 ± 2,95	2,57 ± 1,92

Table 1. Apoptosis in MCF10A cells and MDA-MB-231 cells represented as percentage of cells (mean ± SEM of three independent experiments) after treatments with UV and ER at 0,1 µM, 1 µM and 10 µM. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.
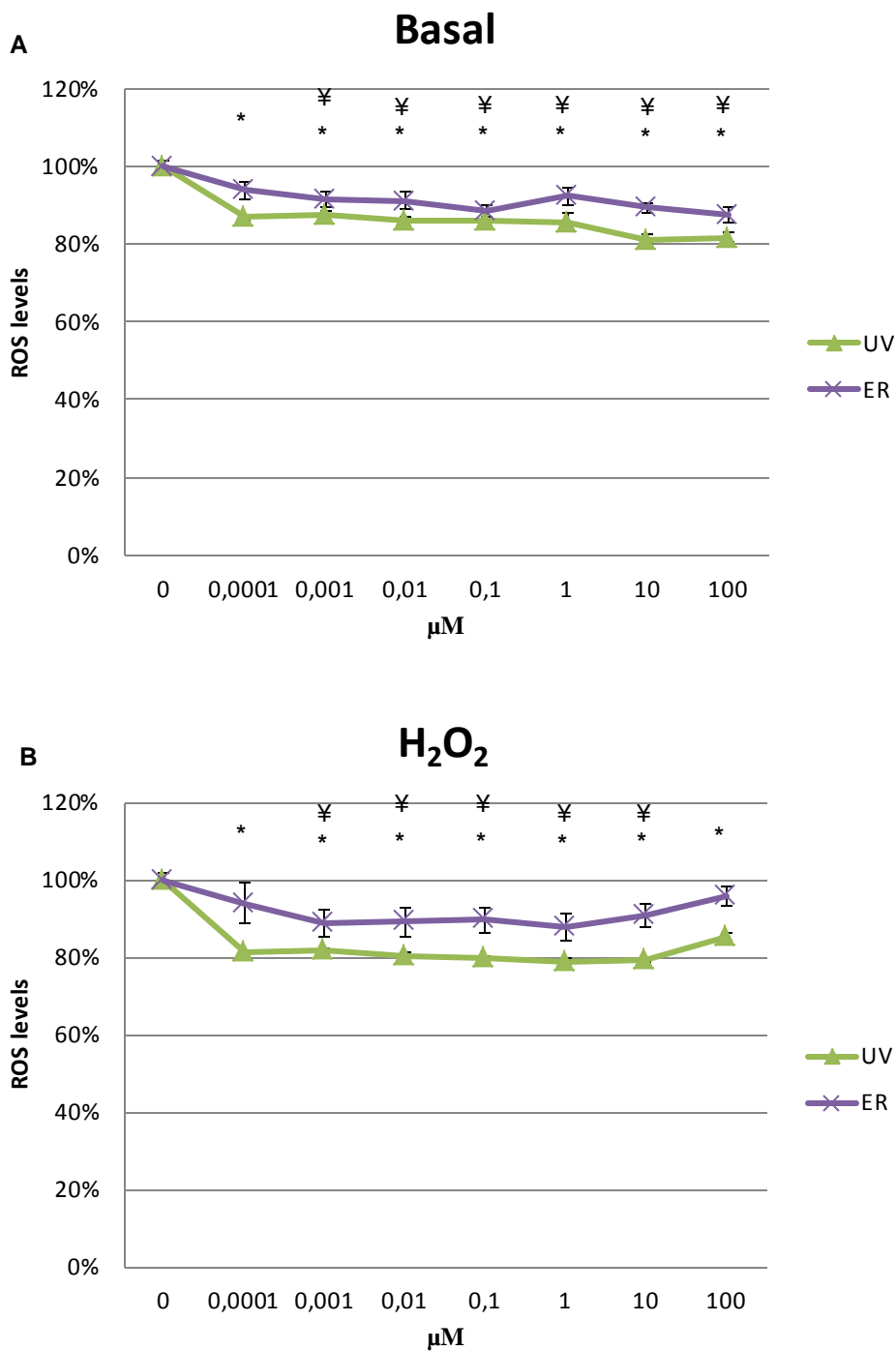
Figure 6. ROS levels of MCF10A in basal state of the cells (A) and after exposure to H_2O_2 (B) with treatments of UV and ER in a range of concentration between 0,0001

μM and 100 μM after 4h. Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.
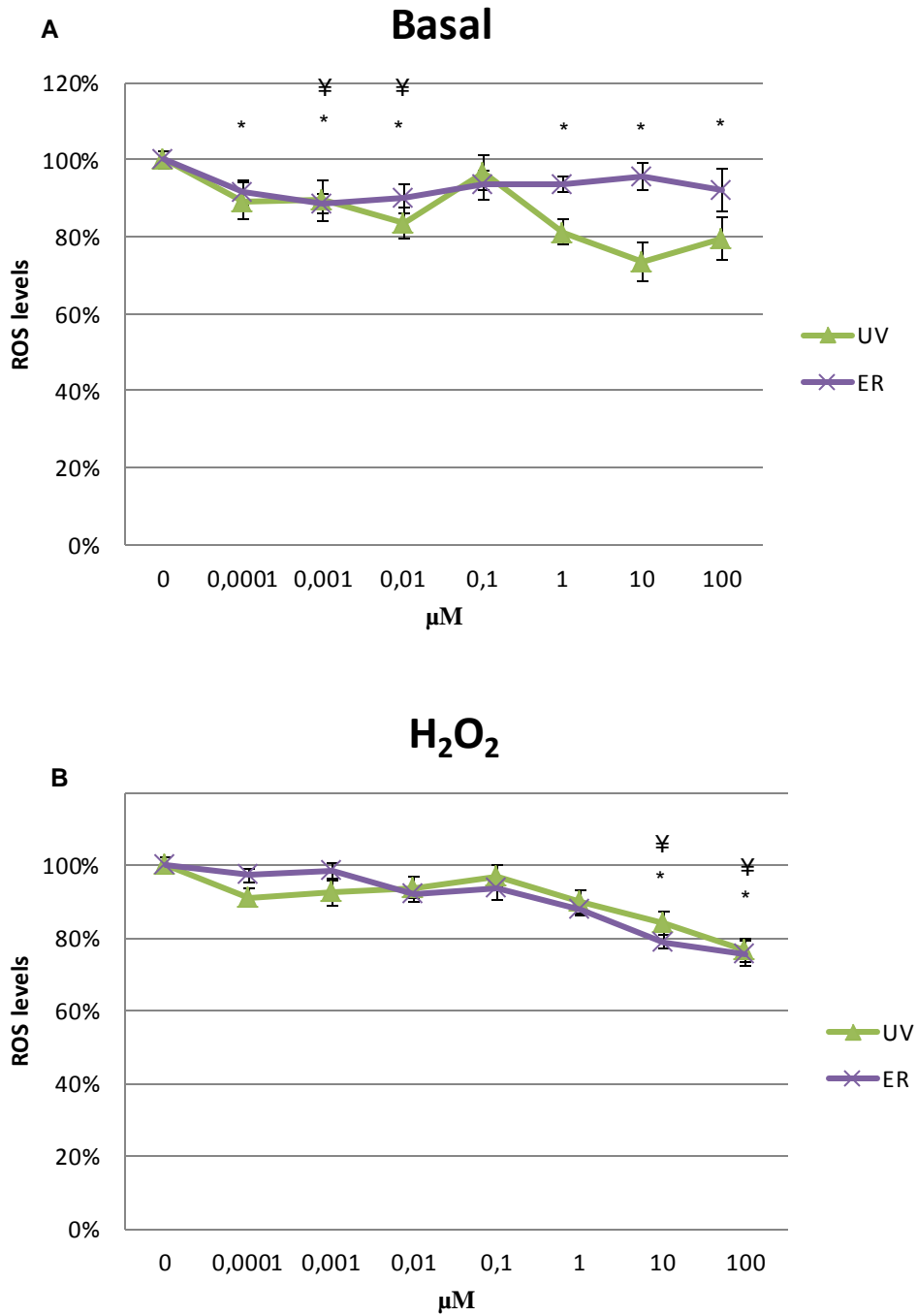
Figure 7. ROS levels of MDA-MB-231 in basal state of the cells (A) and after exposure to H_2O_2 (B) with treatments of UV and ER in a range of concentration between 0,0001 µM and 100 µM after 4h. Numerical values are presented as the mean ±SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at p<0,05.
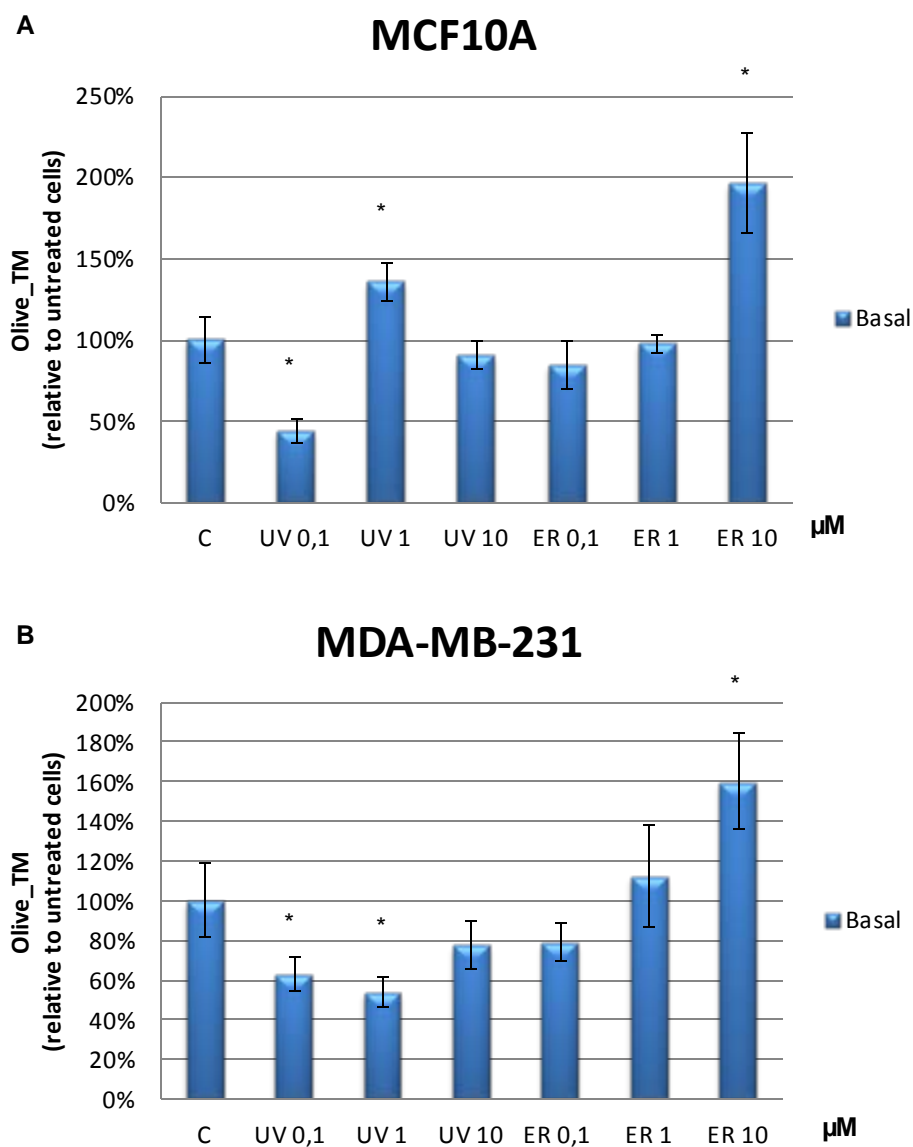

**Figure 8.** DNA damage represented as Olive_TM of MCF10A cells (A) and MDA-MB-231 cells (B) in basal state after treatments with UV and ER at 0,1 µM, 1 µM and 10 µM for 4h. Olive_TM values are represented by mean ±SEM of three independent

experiments. Significant differences were determinate relative to control untreated which was defined as 100% at *p<0,05.
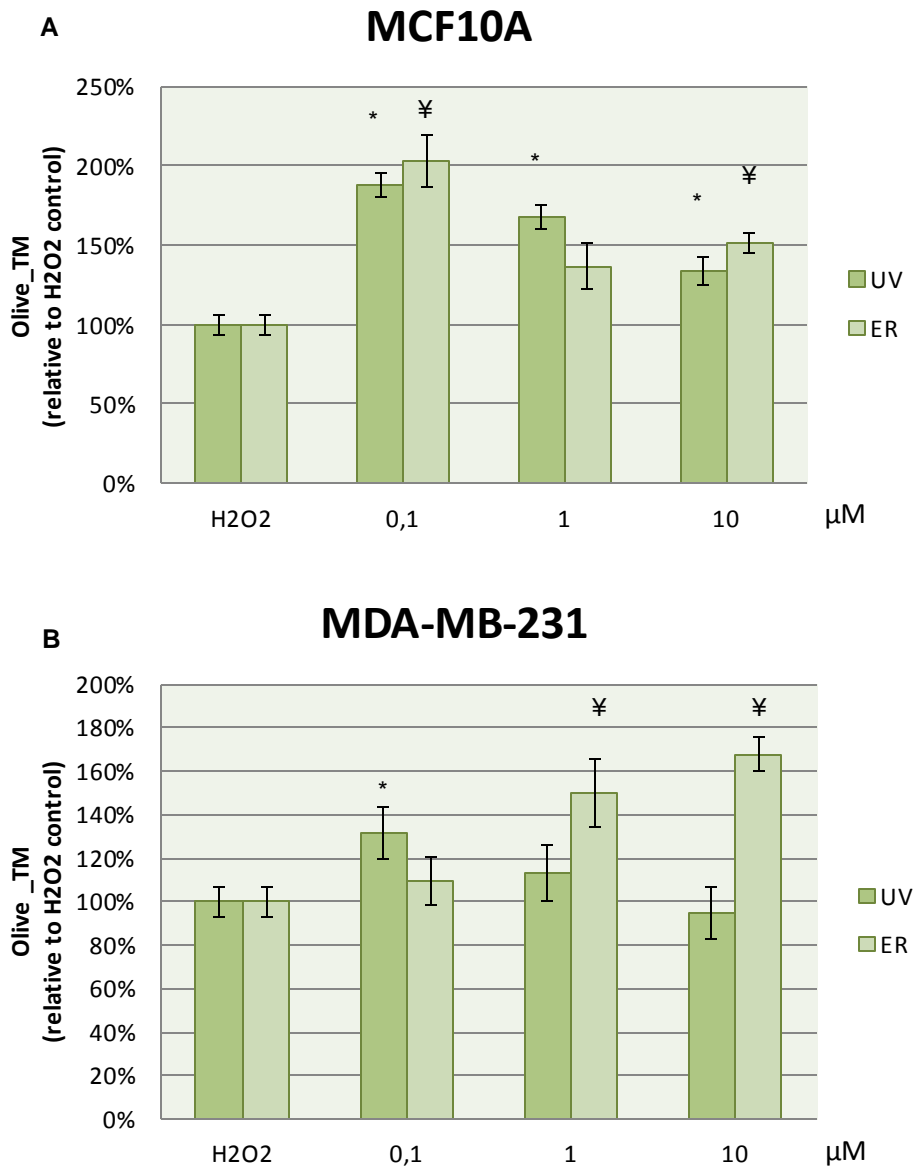
Figure 9. DNA damage represented as Olive_TM of MCF10A (A) and MDA-MB-231 (B) after treatments with UV and ER at 0,1 μM, 1 μM and 10 μM for 4h and after an induced oxidative damage with H_2O_2. Olive_TM values are represented by mean ±SEM

of three independent experiments. Significant differences were determinate relative to H_2O_2 control which was defined as 100% at *p<0,05.

Trabajo experimental y resumen global de los resultados

3. Maslinic acid improves natural immune response through inhibition of chronic inflammation.

Trabajo enviado a la revista "Evidence-Based Complementary and Alternative Medicine".

- Índice de Impacto: 2.175
- Categoría perteneciente: Medicina complementaria e integrativa
- Cuartil en la categoría: Q2
- Rango en la categoría (2013): 6/22
- Fecha de envío: 19-09-2014

Maslinic acid improves natural immune response through inhibition of chronic inflammation.

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Abstract

The inflammatory process is involved in the genesis and evolution of different diseases like obesity, cardiovascular disease and even cancer. Macrophages are immune cells that play a central role in inflammation. In addition, it is known that they can regulate some stages of cancer development. Macrophages can polarize into M1 or M2 functional phenotype, depending on the cytokines present in the tissue microenvironment. On the other hand, triterpenes found in virgin olive oil are described to present different properties, such as antitumoral and anti-inflammatory activity. The present study was designed to elucidate if the four major triterpenes found in virgin olive oil (oleanolic acid, maslinic acid, uvaol and erythrodiol) are able to enhance M1 response that in fact represents an important defense mechanism against the cancer. Our results indicate that maslinic acid shows a more potent action in order to modulate the inflammation response. It increases the recruitment of other macrophages at the site of inflammation by the production of IL-8, IL-1α and IL-1β; it promotes M1 response through the increased synthesis of IFN-γ; and finally, it does not significantly modify the levels of NFκβ or NO. Overall, the results show that maslinic acid inhibits the progress of chronic inflammation.

Introduction

It is well known that patients with chronic inflammatory diseases are at a much higher risk of developing cancer. In 1863, Virchow hypothesized a link between inflammation and cancer based on the presence of leukocytes in neoplastic tissue [1]. In fact, the innate immune system as a first line of defense, mediates the process of inflammation. In vitro and in vivo evidence implicate inflammation in altering multiple pathways related to cancer development [2]. Since the evidence shows that immune cells are able to regulate almost every stage of cancer development, it would be interesting to explore molecular mechanisms that had the potential to modulate the immune response in order to reduce risks.

Macrophages play a central role in both, the development and maintenance of the inflammatory response. Furthermore, macrophages represent the predominant cell type of the innate immune response found within tumors and are known as tumor-associated macrophages (TAMs) [4]. For example, breast cancer is characterized by having a large population of TAMs. Additionally, TAMs release factors to decrease the local pro-inflammatory antitumor response, suppressing it and providing a means of escape of the tumor cells [3].

These cells are able to synthesize a wide variety of molecules such as, pro-inflammatory cytokines, anti-inflammatory cytokines, or proteins related with the whole inflammation process such as nuclear factor kappa beta (NF-κβ), which in turn can trigger the synthesis of pro-inflammatory cytokine IL-1β. The activation of NF-κβ into inflammatory response could be induced by other pro-inflammatory molecules like nitric oxide or by feedback of pro-inflammatory cytokines (IL-1, TNF-α) [4]. TAMs come from monocytic precursors from the blood and undergo specific differentiation depending on local cues in the tissue. Macrophages can be differentiated into either M1 macrophages or M2 macrophages. M1 macrophages activate type 1 helper T cells (Th1), have the ability to kill pathogens and are tumoricidal. On the other hand, M2 macrophages activate type 2 helper T cells (Th2), are involved in wound healing where they downregulate the inflammatory reactions, promote angiogenesis, and have a weak tumoricidal capability [5]. TAMs which are often abundantly present in malignant tumors, share many common features with the alternative activated anti-inflammatory macrophages (M2). Furthermore, these cells have been shown to enhance tumor progression by promoting tumor invasion, migration and angiogenesis. It is well established that depending on the stage of tumor and the kind of macrophage population present, the tumor growth could be affected. As a matter of fact, in solid tumor a high M1/M2 ratio is associated with an

improved survival [6]. Thus, it seems clear that a predominance of M1 macrophage response is beneficial for modulate the inflammatory response in carcinogenesis; they could act against cancer promoting a Th1 cytotoxic response.

On the other hand, oleaonolic acid (OA), maslinic acid (MAS), uvaol (UV) and erytrodiol (ER) are the main pentacyclic triterpenes found in both olive fruit and virgin olive oil [7], the principal source of fat on Mediterranean diet [8]. The benefits of the Mediterranean diet are well known, and are described in many articles [9, 10]. It is believed that virgin olive oil is the responsible of these beneficial effects, among other things, because of its anticarcinogenic properties and protection against DNA damages [11-14]. The main triterpenes of olive oil have been described to possess cardioprotective activities [15, 16], antioxidant effects, antitumor properties [7] and anti-inflammatory activity [17-20]. But so far, we do not know the effects of triterpenes on the innate immune response.

Accordingly, the present study was designed to investigate the effects of the major triterpenes present in virgin olive oil on innate immune response and more precisely the effect in M1 macrophage response in order to elucidate if these compounds are able to enhance M1 response that allow enhance the anti-tumor response. For this purpose, we used an *in vitro* cell experimental model widely accepted to study the inflammatory response.

Methods and Materials

Chemicals

Erythrodiol (ER) CAS [545-48-2], uvaol (UV) CAS [545-46-0], and oleanolic acid (OA) CAS [508-02-1] (purity ≥97, 98,5, and 99%, respectively) were purchased from Extrasynthese (Genay, France). Maslinic acid (MAS) CAS [4373-41-5] (purity >98%) was obtained from Cayman Chemical (Ann Arbor, MI, USA). The following were purchased from Sigma-Aldrich Co. (St Louis, MO, USA): Hepes solution; Sodium Pyruvate solution; Non-Essential Amino Acids mixture 100× (NEAA); Lipopolysaccharides from Escherichia coli 055:B5 (LPS); 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium salt) (purity ≥90%); N-Methylphenazonium methyl sulfate (PMS) (purity ≥98%); Phorbol 12-myristate 13-acetate (PMA) (purity ≥99%); phosphate buffer saline (PBS); sodium chloride (NaCl) (purity ≥99,5%); L-Arginine (L-Arg) (purity 98.5-101.0%) suitable for cell culture and Triton X-100. Fetal Bovine Serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, Austria). Minimum essential medium with Eagle's salts (MEM) and Phenol-Red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from Gibco® Life Technologies Ltd (Paisley, UK). Methanol dry (max 0,005%), Magnesium Chloride (50% MgCl2 powder QP) (MgCl2) and ethanol

absolute PRS was purchased from Panreac Quimica S.L.U. (Barcelona, SPAIN). TrypLE Express was obtained from Invitrogen (Eugene, OR, USA). β-Mercaptoethanol was purchased from Applichem GmbH (Darmstadt, GERMANY). PIPES (98,5+%) was obtained from Acrōs Organics (Geel, BELGIUM). Culture plates were obtained from Starlab (Hamburg, GERMANY). NFκβ p65 (F-6) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RayBio® Human Cytokine Antibody Array (Human Inflammation Array I) was purchased from RayBiotech, Inc. (Norcross, GA, USA).

Cell line and culture conditions

The THP-1 (human acute monocytic leukemia) cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The THP-1 monocytes were maintained at 37°C in a humidified atmosphere under 5% CO_2 in MEM supplemented with 10% FBS, 1% hepes buffer, 1% sodium pyruvate, 1% NEAA and 0,05 mM 2-mercaptoethanol. THP-1 cells were sub-cultured at least twice per week, and discarded and replaced by frozen stocks after 25 passages for achieving an optimal condition of growth.

Macrophages differentiation was induced by treating THP-1 cells ($1x10^6$ cells/ mL) for 24 h with 50 nM of PMA followed by a period of further culture without PMA. PMA-differentiated THP-1 cells ($1,5 \times 10^5$ cells/mL), were stimulated for 24 h with LPS (1 μg/mL) for acquire the M1 phenotype macrophage, and it was followed by oleanolic acid (OA), maslinic acid (MAS), uvaol (UV) or erythrodiol (ER) treatment at 1, 10 and 100 μM for 4 h. All the assays were conducted under these conditions except for those specified below.

Cytotoxicity Assay

THP-1 cells survival, measured as the cellular growth of treated cells versus untreated controls, was carried out using an XTT-based assay according to Scudiero et al. with some modifications [21]. Briefly, cells were seeded into 96-well culture plates in a total volume of 100 μL per well. After overnight incubation to allow cell attachment, 100 μL of fresh medium was added containing increasing concentrations from 0,001 μM to 100 μM of OA, MAS, UV or ER for another 24 h. Thereafter, cells were incubated with XTT in Phenol-Red free RPMI medium for 3 h, and absorbance was measured at 450nm wavelength (620nm as reference) in a plate reader (TECAN GENios Plus). Viability was calculated using the formula:

% viable cells = [(A treated cells) / (A control)] x 100

Where A is the difference in absorbance between optical density units ($A = OD_{450} - OD_{620}$). All measurements were performed in quadruplicate and each experiment was repeated at least three times.

RayBio® Human Cytokine Antibody Array in M1 state THP-1 macrophages

Differentiated THP-1 cells were stimulated with LPS (1 μg/mL) at 24 h. After that, triterpenes treatments were realized. Then, supernatants were isolated and processed according to manufacturer instructions. Arrays membranes were directly detected using a chemiluminescence imaging system (FluorChem E System, ProteinSimple) for achieve production levels of the following cytokines/proteins: eotaxin, eotaxin-2, interleukin 1 alfa (IL-1 α), interleukin 1 beta (IL-1β), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4),interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8),interleukin 10 (IL-10), interleukin 11 (IL-11), interleukin 12 p40 (IL-12 p40), interleukin 12 p70 (IL-12p70), interleukin 13 (IL-13), interferon-gamma (IFN-gamma), granulocyte colony-stimulating factor (GCSF), granulocyte macrophage colony-stimulating factor (GMCSF), chemokine CCL-1 (I-309), metallopeptidase inhibitor 2 (TIMP-2).

Data were analyzed with the RayBio® Human Inflammation Antibody Array 1 Analysis Tool (Cat # SO2-AAH-INF-1). Data are expressed as the relative intensity (RI) between the sample and the LPS stimulated control [RI = (AU $_{sample}$/AU $_{control}$)]. Where AU is the chemiluminescents arbitrary units acquired by the chemiluminescence imaging system.

The results are showed like the fold change (ratio of the sample value respect to the control, which was set as 1) and data are classified in groups depend on their fold change.

Flow cytometry for NFκβ detection in M1 state THP-1 macrophages

After stimulation of differentiated THP-1 cells with LPS (1μg/mL) and the triterpenes treatments, cells were harvested with TrypLE Express and centrifugated at 300 xg at 4ºC for 10 min. The supernatant was detached and 150 μl of cold methanol was added to the pellet. Cells were incubated 10 min at -20ºC. Then, cells were washed with cold PBS, and 1 mL of PIPES buffer (PIPES 10 mM, NaCl 0,1M, $MgCl_2$ 2mM, 0,1% Triton X100 on PBS) was added to each tube. Cells were incubated at room temperature (RT) for 10 min. After that, cells were washed and suspended in anti-NFκβ antibody buffer (1 μg/ 100 μl) on darkness at RT for 30 min. Later, cells were washed and analysed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). Mains of the samples were calculated using the FlowJo program (v5.7.2). Each experiment was repeated at least three independent times. Data were showed as the fold change respect to control.

NO production in M1 type THP-1 macrophages

Nitric oxide (NO) production was measured according to F. Amano with some modifications [22]. Differentiated THP-1 cells (5x10^5 cells/mL) were seeded on a 12-well- plate, and treated with OA, MAS, UV or ER at 0.1, 1 and 10 μM throughout 3 h.

Then, LPS (1 μg/mL) and L-Arginine (L-Arg) at 10 mM were added to the wells and incubated for 24 h. Supernatants were collected and incubated with ethanol absolute 30 min. at -20ºC. Later, supernatants were centrifuged at 10.000 xg at 4ºC for 10 min and finally, they were aliquoted. NO production was analysed by a NO analyser (NOA 280i de SIEVERS, GE Water and Process Technologies, Pennsylvania, USA). Data were expressed as the percentage of concentration measured relative to untreated control, which was set as 100%.

Statistical analysis

For all the assays except for cytokine antibody array, data are displayed as the mean of at least three independent experiments (±SEM); for cytotoxicity assay, 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 <0.05 was considered to be statistically significant. These statistical analyses were performed using Statgraphics Centurion XVI statistical software (Statpoint Technologies, Inc., Warranton, VA).

Results

Cell cytotoxicity effects

Cell survival was determined by the XTT assay. THP-1 cells were differentiated and exposed to increasing concentrations (from 0,001 μM to 100 μM) of OA, MAS, UV and ER for 24 h. Our results showed that the four triterpenes assayed decreased significantly cell viability at 100 μM, whereas at lower concentrations, they did not show cytotoxic effects (Figure 1).

Effects on RayBio® Human Cytokine Antibody Array

Production of inflammation-related proteins was realized on THP-1 macrophages cells stimulated with LPS (1 μg/mL) for achieve a M1 phenotype. All the inflammation-related proteins were significantly increased on LPS stimulated cells respect to unstimulated control cells (data not shown).

M1/M2 polarization related cytokines

After triterpenic treatments we observed that IFN-γ, which leads to M1 polarization, presented high levels respect to control at MAS 1 and 10 μM and ER 1 μM (Figure 2a and 2b). For the rest of compounds, IFN-γ production levels were similar to control (Figure 2c and 2d). While IL-4, which leads to M2 polarization, showed low levels with MAS 1 μM treatment and being absent with MAS 10 μM and ER 1 and 10 μM (Figure 2a and 2b). IL-10 did not show any significant differences respect to control. In the other compounds tested there are no differences respect to control in their production (Figure 2c and 2d). At the concentration of 100 μM, the most of

compounds have strong differences respect to the control, but it might be due to the cytotoxic effects that they exert at elevated concentrations.

Macrophages recruitment-related cytokines and pro-inflammatory cytokines

Cytokines related with macrophages recruitment such as IL-8, IL-1 alpha and IL-1 beta appeared increased in treatment at 10 µM of MAS in THP-1 M1 phenotype cells. The production of IL-6 cytokine was high with the same concentration (Figure 3). For the rest of compounds only the IL-8 cytokine production was increased for UV 10 µM, and IL-1 alpha for ER 10 µM. The IL-6 cytokine levels appeared increased in all the treatments at 10 µM and for ER at 1µM (Figure 4).

For the rest of cytokines and proteins related with inflammation the signal were closed to background (data not shown).

Effects on NF-κβ production

Detection of NF-κβ (p65) was realized by flow cytometry on differentiated THP-1 cells stimulated with LPS 24 h and treated with 1, 10 and 100 µM of OA, MAS, UV or ER triterpenes. There were not statistical differences between control and samples (Table 1).

Effects on NO production

NO production was measured on M1 phenotype THP-1 macrophages at 0.1, 1 and 10 µM of OA, MAS, UV or ER. Although any treatment exhibited a statistically significant variation compared with the LPS stimulated control, a slight increase of NO production was observed at MAS 1 and 10 µM and ER 10 µM and a decrease at OA 10 µM and ER 1 µM (Figure 5). LPS stimulated control showed statistical differences respect to unstimulated control (data not shown).

Discussion

It is known that THP-1 cell line has a closely gene expression to primary macrophages, derived from peripheral blood mononuclear cells, in contrast to other monocytes cell lines like U937 [23]. Furthermore a PMA differentiation of THP-1 cells drives to a differentiated macrophage phenotype that seems very nearby to monocyte-derived human macrophages [24]. Analysis of primary macrophages in culture will always provide more truthful information about inflammation response, but these primary cultures are also difficult to culture in the quantities required to allow biochemical analysis. Thus, PMA differentiated and LPS stimulated THP-1 cells represent a useful experimental model to study the inflammatory response and their modulation after food compounds treatments [25]. Moreover, recently it has been described the consequent M1 phenotype polarization that LPS promotes in THP-1

macrophages [26]. Thus, THP-1 macrophages are the best option for study the *in vitro* effects of certain compounds in macrophages M1.

Attending to this, we have used this experimental model to study *in vitro* effects of OA, MA, UV and ER in M1 macrophages. We polarized THP-1 macrophages into the M1 stage by LPS treatment and promoted an inflammatory microenvironment.

Macrophages constitute an extremely heterogeneous population, which polarize into distinct macrophages types, mainly identified as M1 (or classically activated) and M2 (or alternatively activated) [27]. Previously, we have described two phenotypically and functionally different populations among splenic macrophages in response to *C. albicans* infection. One of them (M2 phenotype) showed high levels of major histocompatibility complex (MHC) class II surface expression and are poorly phagocytic. The other one (M1 phenotype), expressed low levels of MHC class II molecules and are highly phagocytic [28]. We suggests that NK cells prime splenic macrophages were phagocytic in naïve BALB/c mice, probably mediated by IFN-γ production, the same signal that monocytes need in tumor microenvironment for polarized to M1 phenotype. Thus, infections as well as cancer could polarize macrophages to M1 or M2 phenotypes depending on the microenvironment signals [6].

M1 macrophages act against tumors producing high amounts of pro-inflammatory cytokines and activating the immune response [27], they are believed to act in the early stages of tumor development. Indeed in nonprogressing or regressing tumors, TAMs are related to a classic macrophage activation M1-like program, characterized by proinflammatory activity, antigen presentation and tumor lysis. Even more a high M1/M2 polarization ratio improved survival in lung carcinoma [6]. In malignant tumors, TAMs resemble M2 phenotype that increases angiogenesis and tumor cell extravasation and growth; they suppress activation of dendritic cells, cytotoxic T lymphocytes and natural killers [29, 30].

The role of M1 in infection is not well documented, but nowadays this population is commonly studied due to the interest in new anticancer therapeutics related with inflammatory conditions that allow tumor growth. Some studies indicate that M1 have a proangiogenic function early in tumorigenesis [31], when the tumor needs blood vessels formation for the growth; this supports the idea of the role that M1 plays in the early stages of breast tumor formation and it seems to be one of the first immune cells present when an inflammatory process is taken place. But, in advanced breast cancers, macrophages resemble the M2 phenotype while M1 phenotype has not been found; this is the reason why TAMs are generally related more to a M2 phenotype than M1. Further, M2 macrophages express changes in several metabolic pathways, controlling the inflammatory response by down-regulating M1-mediated functions. It seems to be clear that the tumor cells are able to produce several signals

that polarized the monocyte to M2, promoting his own growth. This preferential polarization is a result of the absence of M1-orienting signals, such us INF-γ or bacterial components in the tumor [27].

We hypothesized that, in established solid tumors, the activation of a M1 response, could be a useful strategy in order to prevent tumor growth.

M1 macrophages are originated from monocytes stimulated by INF-γ and microbial stimuli such as LPS, and they are characterized by a high pro-inflammatory cytokines production and consequent activation of polarized type 1 T cell response, cytotoxic activity against microorganism and neoplastic cells, and good capability as antigen-presenting cells (APCs). Our results show that MAS at the lowest and medium concentrations and ER at lowest concentration increased the production of INF-γ in M1 polarized THP-1 macrophages (Figure 2a and 2b). By this way, M1 macrophages could mediate and control their own response activating monocytes to M1 instead of M2, for example in carcinogenesis. This increase of INF-γ production would be a pro-inflammatory signal to monocytes that arrive to the inflammation site and they could be polarized to M1 phenotype, making more efficient the recognition of neoplastic cells and mediating a Th1 cells response, which in fact is also activated by INF-γ.

It has been recently discussed the idea of M2 to M1 macrophages switch at the site of the tumor by INF-γ induction such as receptor-mediated activation signals to promote tumor regression [6]. With MAS and ER, it could be the own M1 polarized macrophages present at this site, who could hypothetically reprogram new M2 macrophages that could show up at the sites of tumor formation.

The macrophages are differentiated like M2 phenotype when monocytes are stimulated with IL-4 and IL-13, with immune complexes/TLR ligands, or with IL-10 and glucocorticoids. They have poor antigen-presenting capability, high anti-inflammatory cytokines production, mediate angiogenesis and wound-healing promotion. In our study, MAS and ER also inhibited IL-4 production at the majority of concentration tested (Figure 2a and 2b), thereby M2 polarization were not able by this way for monocytes recruited later at the inflammation site. M2 polarization by IL-10 is not possible because production of this cytokine in these macrophages was poor or even absent (Figure 2a).

M2 macrophages are not capable to produce IL-6 [32], which continues with the pro-inflammatory response, but MAS (Figure 3) and UV 10 μM (Figure 4) treatments showed to enhance IL-6 production. Even more, MAS at 10 μM and UV at 10 μM showed levels of IL-8 production slightly higher than the control (Figure 3 and 4). It is known the role of IL-8 cytokine in the monocyte recruitment as well as CXCL12, chemokine which precursors are IL-1α and IL-1β, interestingly increased in MAS at 10 μM (Figure 3).

Consequently, apart from strengthen the pro-inflammatory response activating monocytes to M1 and prevent the M2 polarization of monocytes at sites of inflammation, these compounds appear to promote the M1 response with the recruitment of more cells that could support the immune response at the inflammation site.

Otherwise, previous studies showed that the deleterious role of macrophages in cancer progression is due to the cooperation of monocytes with cancer cells, and this indicates a functional plasticity and in situ macrophages switch; however the biological mechanisms for this switch of M1 to M2 remains controversial. Some authors highlight that activation of NF-κβ promotes the presence of immunosuppressive M2 phenotype [33]. In order to assess the possible implication of triterpenes in promote the NF-κβ induction, we evaluate its production by the M1 phenotype THP-1 macrophages. It is important to note that a high increase of NF-κβ expression may lead to an aggravated inflammatory response that could guide to a consequent chronic inflammation [33]. In the present study the levels of NF-κβ were unaltered in all samples, so these M1 macrophages treated with triterpenes did not show to promote chronic inflammation.

Furthermore, nitric oxide (NO) production by NOS (nitric oxide synthase) supports this point. At normal levels, NOS acts like protector against injury, but at elevated levels in the tissue, it has been described like an inflammatory enzyme that promotes carcinogenesis [34]. We study the levels of NO after the treatment with triterpenes and there is no statistically significant change in their production compared to the control (Figure 5).

It is important to note that at the highest concentration, these triterpenes are cytotoxic for THP-1 macrophages (Figure 1) but the effects of these triterpenes in M1 polarized macrophages focus at low concentrations.

We conclude that, among the triterpenes present in virgin olive oil, maslinic acid shown a more potent action in order to prevent M2 polarization in an inflammation process, fortifying M1 response. This assertion is based on the following findings:

1. Maslinic acid could increase the recruitment of other macrophages at the site of inflammation by the production of IL-8, IL-1α and IL-1β.

2. Maslinic acid promoted M1 response, through the increased synthesis of IFN-γ.

3. Maslinic acid did not seem to promote chronic inflammation. It did not modify the levels of NF-κβ or NO.

Previously, we have described the activity of these triterpenes in a breast cancer cell line [7] and now we describe their action in M1 phenotype THP-1 macrophages.

Although a study more deeply is needed for assess the action of these macrophages treated with triterpenes at sites of tumour, this study shows that these triterpenes could be useful natural compounds that modulate inflammation process in cancer development and may play a role against the tumor growth. Furthermore, there are natural compounds which could be intake with the diet and this way, protect against tumor development at sites of inflammation without toxic effects.

Nevertheless extreme caution should be applied in the extrapolation of *in vitro* results to potential human effects. Further studies are needed to confirm the mechanism of action of these compounds and the protective actions versus inflammatory response that they could have *in vivo*.

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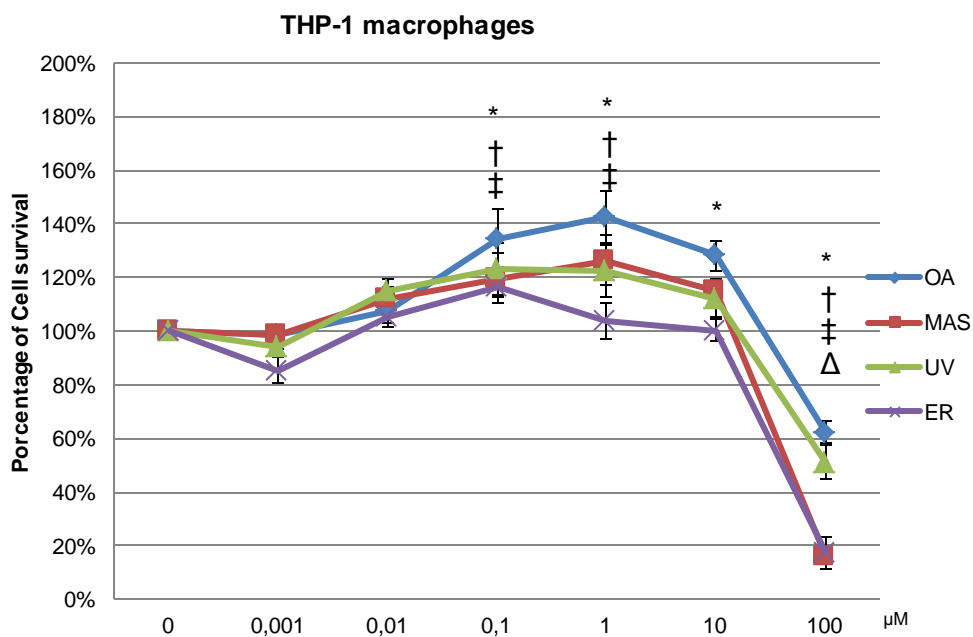
Figure 1. Effects of triterpenes on THP-1 macrophages survival. Cells were differentiated and treated with 0,001-0,01-0,1-1-10-100 μM of oleanolic acid (OA*), maslinic acid (MAS†), uvaol (UV‡) and erythrodiol (ERΔ) at 24 h.

(*)(†)(‡)(Δ) Statistically significant difference compared with control (p<0.05).

a)

MAS
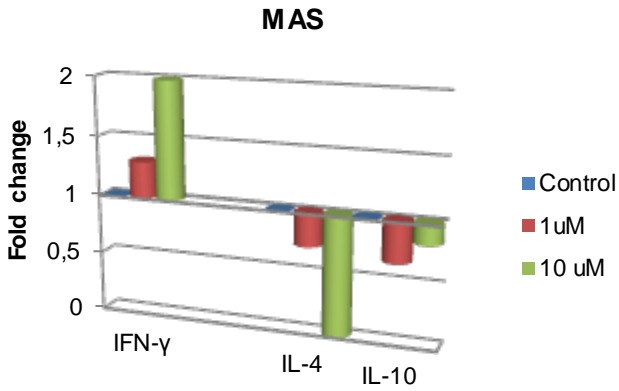
	MAS				
Activity	**Cytokine**	**Control**	**1 μM**	**10 μM**	**100 μM**
M1 polarization	**IFN-γ**	C	*D*	*E*	C
M2 polarization	**IL-4**	C	*B*	*A*	A
	IL-10	C	*B*	C	B

b)

ER
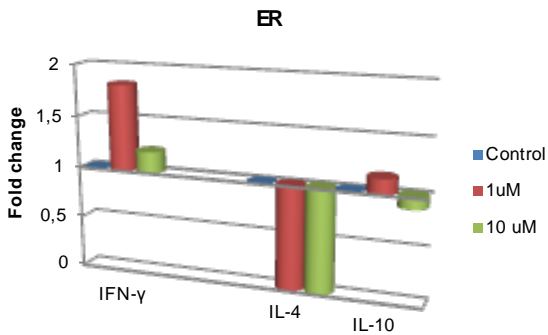
	ER				
Activity	**Cytokine**	**Control**	**1 μM**	**10 μM**	**100 μM**
M1 polarization	**IFN-γ**	C	*E*	C	C
M2 polarization	**IL-4**	C	*A*	*A*	A
	IL-10	C	C	C	C

c)
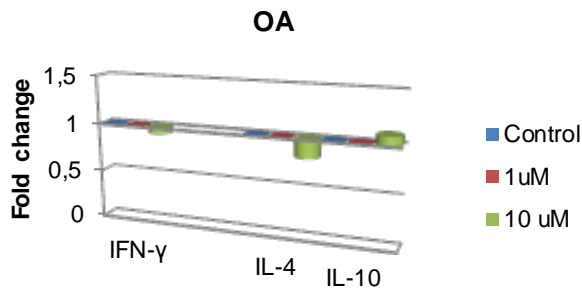
OA

	OA			
Activity	**Cytokines**	**Control**	**1 μM**	**10 μM**	**100 μM**
M1 polarization	**IFN-γ**	C	C	C	D
M2 polarization	**IL-4**	C	C	C	A
	IL-10	C	C	C	D

d)
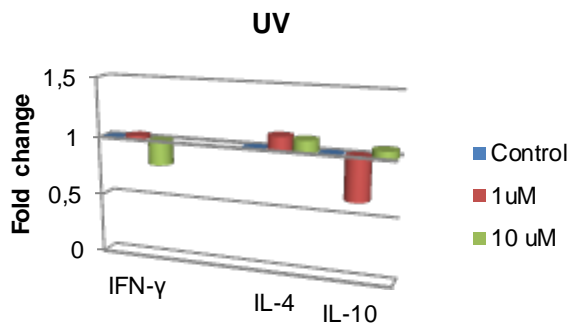
UV

	UV			
Activity	**Cytokines**	**Control**	**1 μM**	**10 μM**	**100 μM**
M1 polarization	**IFN-γ**	C	C	C	D
M2 polarization	**IL-4**	C	C	C	A
	IL-10	C	*B*	C	C

	Fold change
A	0-0,25
B	0,25-0,75
C	0,75-1,25
D	1,25-1,75
E	1,75-2,75
F	2,75-4,25

Figure 2. M1 polarization related cytokines production *versus* M2 polarization related cytokines production in M1 polarized THP-1 macrophages treated with MAS (a), ER (b), OA (c) and UV (d) at 1, 10 and 100 μM. Results are expressed as the fold change in RI (relative intensity) related to stimulated control which was set as 1.

MAS
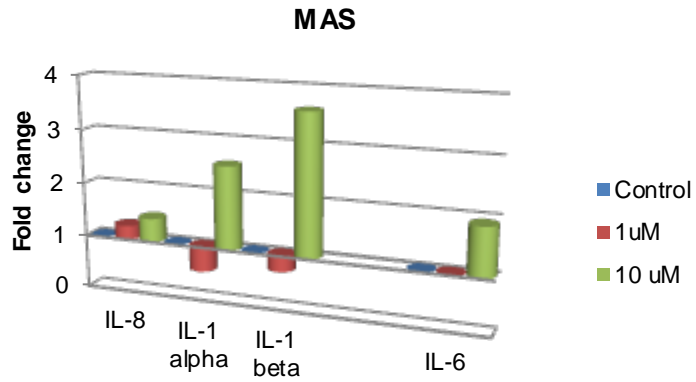
Activity	Cytokines	Control	1 μM	10 μM	100 μM
Macrophages recruitment	**IL-8**	C	C	*D*	C
	IL-1 alpha	C	B	*E*	F
	IL-1 beta	C	B	*F*	F
Pro-inflammatory	**IL-6**	C	C	*E*	B

Figure 3. Production of cytokines related with macrophages recruitment and other pro-inflammatory cytokines in M1 polarized macrophages treated with MAS at 1, 10 and 100 μM.

a)

OA
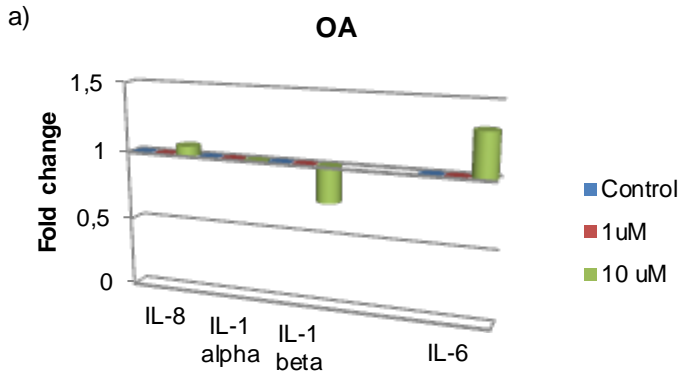
	OA			
Activity	**Cytokines**	**Control**	**1 µM**	**10 µM**	**100 µM**
Macrophage recruitment	**IL-8**	C	C	C	C
	IL-1 alpha	C	C	C	D
	IL-1 beta	C	C	B	B
Pro-inflammatory	**IL-6**	C	C	*D*	B

b)

UV
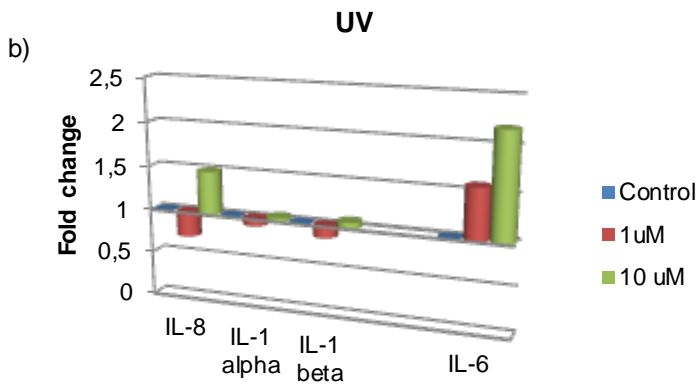
	UV			
Activity	**Cytokines**	**Control**	**1 µM**	**10 µM**	**100 µM**
Macrophage recruitment	**IL-8**	C	B	*D*	D
	IL-1 alpha	C	C	C	D
	IL-1 beta	C	C	C	E
Pro-inflammatory	**IL-6**	C	D	*E*	B
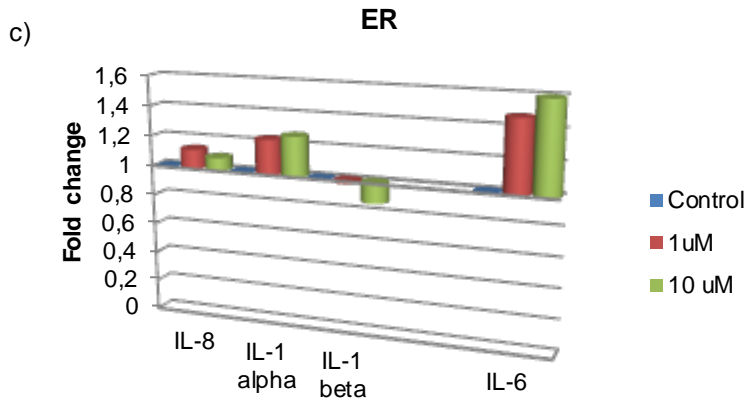
c)

				ER		
Activity	**Cytokines**	**Control**	**1 µM**	**10 µM**	**100 µM**
Macrophage recruitment	**IL-8**	C	C	C	C
	IL-1 alpha	C	C	*D*	E
	IL-1 beta	C	C	C	F
Pro-inflammatory	**IL-6**	C	*D*	*D*	A

Figure 4. Production of cytokines responsible of macrophage recruitment produced by M1 polarized THP-1 macrophages, treated with OA (a), UV (b) and ER (c) at 1, 10 and 100 µM.
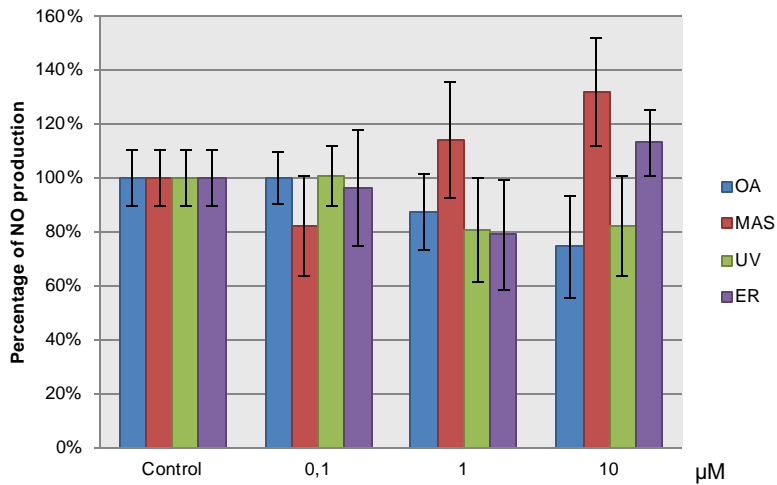
Figure 5. NO production of M1 polarized THP-1 macrophages treated with 0.1, 1 and 10 µM of OA, MAS, UV and ER. Data are expressed relative to control without treatment, which was established as 100%. Not statistical differences were found.

Treatment	Concentration	Fold Change
Control		0,00
OA	*1 μM*	0,32
	10 μM	-0,26
	100 μM	-0,16
MAS	*1 μM*	-0,04
	10 μM	0,01
	100 μM	0,16
UV	*1 μM*	0,11
	10 μM	0,21
	100 μM	0,02
ER	*1 μM*	0,10
	10 μM	0,01
	100 μM	-0,01

Table 1. NFκβ production by M1 polarized THP-1 macrophage cells treated with OA, MAS, UV and ER at 1, 10 and 100 μM along 4 h, measured by flow cytometry. Data are expressed like fold change in NFκβ production respect to control. Not statistical differences found.

Trabajo experimental y resumen global de los resultados

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

Trabajo enviado a la revista "Journal of Agricultural and Food Chemistry".

- Índice de Impacto: 3.107
- Categoría perteneciente: Tecnología y ciencia de la Alimentación
- Cuartil en la categoría: Q1
- Rango en la categoría (2012): 13/123
- Fecha de publicación: 22/11/2013

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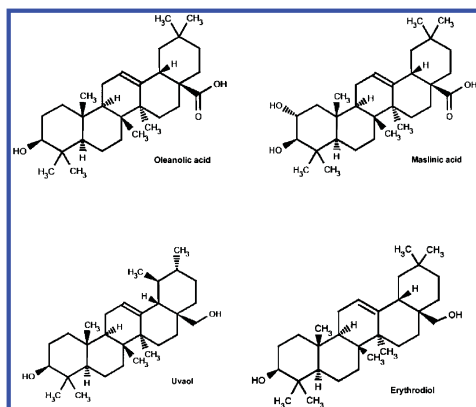
Figure 1. Chemical structures of oleanolic and maslinic acids and uvaol and erythrodiol dialcohols of olive oil.

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 $0.29 \pm 0.26\ \mu M$ 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 knockout 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 myocyte 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 (HU-VECs).[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).
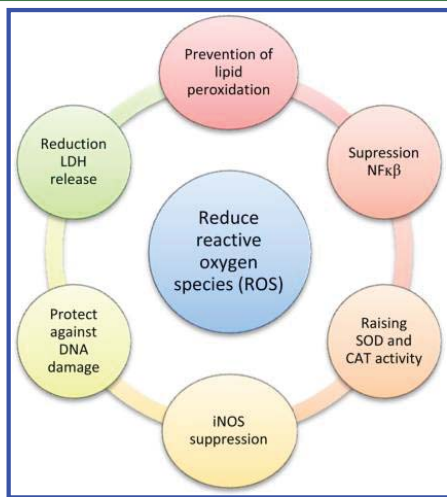

Figure 2. Oxidative mechanisms affected by triterpenes.

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 lip-opolysaccharide (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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5. Resumen global de los resultados

1. Trabajo Experimental titulado "Oleanolic acid selectivity inhibits proliferation of highly invasive human breast cancer cells and protects against oxidative DNA damage in normal mammary epithelial cells".

1.1 Acción antitumoral de los compuestos ácido oleanólico y ácido maslínico en la línea tumoral humana de mama MDA-MB-231.

Los resultados obtenidos mostraron que a la más alta concentración (100 μM) tanto el ácido oleanólico (OA) como el ácido maslínico (MA) promovían la muerte celular en las células de la línea tumoral altamente invasiva de mama, MDA-MB-231 (figura 1c). Y tras 48 y 72 h de tratamiento ambos compuestos lograban inhibir la proliferación de manera dosis dependiente (fig 4).

El ciclo celular se vio afectado tras el tratamiento con OA, aumentando el porcentaje de células en la fase G2/M del ciclo en todas las concentraciones ensayadas, aunque únicamente eran estadísticamente significativas la mayor y la menor concentración (tabla 1). Sin embargo, en el estudio de apoptosis, ninguno de los compuestos ensayados promovía la muerte celular programada en estas células tumorales altamente invasivas (tabla 2).

En el estudio de las EROs (especies reactivas de oxígeno), el estrés basal intracelular tras el tratamiento con OA, era mayor que en el control a las concentraciones 0,001 y 100 μM. Al inducir un aumento del estrés oxidativo intracelular con la adición de peróxido de hidrógeno (H_2O_2), los niveles de estrés eran mayores en las células tratadas con OA (un 30% más a 1 μM) que en las no tratadas (fig. 7b). Tras el estudio de la actividad de la enzima CAT (catalasa) se apreció un ligero descenso de actividad en las células tratadas con OA (fig. 8c), probablemente debido a una acción inhibitoria directa o indirecta del OA, que se reflejó en un aumento del estrés oxidativo intracelular. Mientras que el MA no parecía afectar al estrés oxidativo interno de la célula tumoral.

Se conoce que la acción de las EROs (especies reactivas de oxigeno) provoca daño al ADN, y ya que estos triterpenos interfieren en el estrés oxidativo de la célula, se pasó a evaluar la influencia de los mismos en el daño al ADN de la célula tumoral. Tras promover daño oxidativo al ADN con H_2O_2, las células tratadas con OA parecían presentar un aumento en la longitud e intensidad de fluorescencia de la cola (fig. 9b), indicando un mayor daño al ADN que aquellas que no habían sido tratadas. Igualmente ocurría con el MA, que provocaba un mayor daño al ADN en comparación a las células control (fig. 9b).

1.2 *Acción preventiva de los compuestos ácido oleanólico y ácido maslínico en la línea humana epitelial de mama MCF10A.*

Los efectos de ambos compuestos en esta línea son citotóxicos a concentraciones altas (100 µM) (fig. 1a), mientras que a bajas los efectos observados apuntaban a un incremento de la proliferación de esta línea a medida que avanzaba el tiempo (fig. 2).

Así pues, se observaron efectos apoptóticos a la más alta concentración (10 µM) (tabla 2), que se correspondían con un alto porcentaje de células en la población Sub-G0/G1 del ciclo celular (tabla 1) tras el tratamiento con OA 10 µM. Aunque el efecto era mucho más acusado con el MA, que aumentó en un 64 % las células que podíamos encontrar en la fase Sub G0/G1 (tabla 1).

Para estudiar si estos compuestos eran capaces de aumentar o disminuir el estrés oxidativo en la célula epitelial, se estudiaron las EROs intracelulares. En este caso, se observó que tanto OA como MA a altas concentraciones producían una bajada del estrés oxidativo con respecto al control, a nivel basal (fig 5a). Pero una vez que se incrementaron los radicales libres intracelulares (con la adición de H_2O_2) el MA incrementaba el estrés dentro de la célula, mientras que aunque estadísticamente no era significativo en la mayoría de las concentraciones, el OA tendió a disminuirlos levemente (fig 5b). Este efecto se correlaciona con los encontrados en la actividad de la CAT, incrementada con el tratamiento de OA (fig. 8a).

En el estudio del daño oxidativo al ADN, se observó que los tratamientos con OA disminuían el daño provocado al ADN de las células mediante la adición de H_2O_2 (fig. 9a), por lo que podríamos pensar que este compuesto podría ser efectivo como protector ante el daño oxidativo al ADN.

2. Trabajo experimental titulado "The differential localization of a methyl group confers to two triterpenes present in the olives a different anti-breast cancer activity".

2.1 *Acción antitumoral de los compuestos uvaol y eritrodiol en la línea tumoral humana de mama MDA-MB-231.*

Los tratamientos con uvaol (UV) y eritrodiol (ER) mostraron citotoxicidad para la línea tumoral humana de mama MDA-MB-231 únicamente a la concentración de 100 µM (fig. 2b). De igual manera ocurrió con la proliferación tras el tratamiento con los

dos compuestos, a todos los tiempos ensayados UV a 100 µM mostró un efecto antiproliferativo, mientras que ER sólo tenía tal efecto a las 72 h tras el tratamiento (fig. 4).

No se observaron efectos en el ciclo celular (datos no mostrados) ni inducción de apoptosis tras los tratamientos (tabla 1).

Tras el estudio del estrés oxidativo intracelular, se observó que tanto UV como ER disminuían los niveles basales de estrés oxidativo (fig. 7a), mientras que cuando se incrementaba el estrés intracelular, estos compuestos sólo lo disminuían a las concentraciones mayores (10 y 100 µM) en las células tumorales de mama (fig. 7b).

Los resultados observados en la electroforesis unicelular alcalina (también conocida como Comet Assay) mostraron que UV disminuía el daño oxidativo al ADN, mientras que ER a 10 µM era capaz de promover daño en el ADN (fig. 8b). Una vez que se indujo un aumento del estrés oxidativo mediante la adición de H_2O_2, el tratamiento con UV a 0,1 µM y ER a 1 y 10 µM causaba daños mayores al ADN celular que el observado en las células sin tratar (fig. 9b).

2.2 *Acción preventiva de los compuestos uvaol y eritrodiol en la línea humana epitelial de mama MCF10A.*

Tras el tratamiento con UV y ER se observó un efecto citotóxico en las células de la línea humana de mama, MCF10A, a altas concentraciones (fig. 2a). Sin embargo, la proliferación no se ve afectada por UV, excepto a 100 µM. De la misma manera ER tiene efectos antiproliferativos solo a 100 µM tras 72h de tratamiento (fig. 3).

El ciclo celular de las células epiteliales humanas se ve afectado tras el tratamiento con ER a 10 µM (fig. 5), donde se detiene el ciclo en la fase SubG0/G1, promoviendo apoptosis a esa misma concentración (tabla 1).

A nivel basal, e incluso con la adición de H_2O_2, ambos compuestos son capaces de prevenir el estrés oxidativo en la línea epitelial (fig. 6).

En el análisis del daño al ADN, se observó que el tratamiento con UV a 0,1 µM reducía el daño al ADN a más de la mitad (56,2%) en las células de la línea epitelial de mama en su estado basal. Un efecto adverso provocaba el ER, incrementando dramáticamente el daño al ADN (fig. 8a). Cuando se inducía un choque oxidativo con H_2O_2, ambos tratamientos incrementaron el daño oxidativo al ADN en la línea epitelial normal humana (fig. 9a).

3. **Trabajo experimental titulado "Maslinic acid improves natural immune response through inhibition of chronic inflammation".**

3.1 Efectos citotóxicos del OA, MA, UV y ER en la línea monocitaria humana THP-1.

Los principales triterpenos presentes en el aceite de oliva virgen (OA, MA, UV y ER) no muestran citotoxicidad en la línea monocitaria humana THP-1, excepto a la mayor concentración utilizada (100 µM)(fig. 1).

3.2 Efectos en la producción de citoquinas relacionadas con la polarización a M1 o M2 del macrófago.

Tras la diferenciación y polarización de la línea THP-1 a macrófagos de tipo M1 se procedió al tratamiento con los cuatro triterpenos. Se observó que el interferón gamma (IFN-γ) aumentaba sus niveles tras el tratamiento con MA y ER a 1µM (fig. 2a y b). Cabe destacar que esta molécula polariza los macrófagos presentes al fenotipo M1; mientras que citoquinas como IL-4 o IL-10 conllevan la diferenciación a M2. IL-4, al igual que IL-10, presentaban niveles muy bajos o inexistentes tras el tratamiento con MA, ER y UV (fig. 2a, 2b, 2d).

A la concentración de 100 µM, todos los tratamientos presentaban diferencias con respecto al control, hecho que puede estar debido a la entrada en apoptosis y/o necrosis del macrófago, ya que se observó citotoxicidad a esa concentración con todos los productos.

3.3 Efectos en la producción de citoquinas proinflamatorias y relacionadas con el reclutamiento de macrófagos.

Entre las citoquinas relacionadas con el reclutamiento de macrófagos encontramos la IL-8, IL-1α e IL-1β, cuyos niveles parecían aumentar tras el tratamiento con MA a 10 µM con respecto a las células sin tratar (fig. 3).

La citoquina IL-6 también aparecía aumentada tras el tratamiento con 10 µM para todos los compuestos (fig. 3).

La producción de IL-8 también fue mayor cuando los macrófagos se trataban con UV a 10 µM (fig. 4b). Mientras que IL-1α aumentaba tras el tratamiento con ER (fig. 4c).

3.4 Efectos en la producción de NFκβ y NO.

La detección de NFκβ no mostró ninguna variación en la producción de esta molécula proinflamatoria tras el tratamiento con los cuatro triterpenos (tabla 1).

Pero en la producción de óxido nítrico (NO), aunque los efectos no fueron estadísticamente significativos, hubo un pequeño aumento tras el tratamiento con MAS a 1 y 10 μM y con ER a 10 μM, mientras que ER a 1μM y OA a 10 μM lograban disminuir los niveles de NO en los macrófagos THP-1 (fig. 5).

4. Trabajo de revisión titulado "Bioactive Properties of the Main Triterpenes Found in Olives, Virgin Olive Oil, and Leaves of Olea europaea".

4.1 Triterpenos.

Los triterpenos principales que podemos encontrar formando parte del aceite de oliva, de las aceitunas e incluso de la hoja del árbol del olivo son dos ácidos oleanólico (OA) y maslínico (MA), y dos dialcoholes llamados uvaol (UV) y eritrodiol (ER).

La estructura química de estos cuatro triterpenos es casi idéntica, sólo se diferencian por poseer o bien un grupo hidroxilo (OH⁻) en carbonos diferentes, o bien un grupo metilo (CH_3). Así, el MA se diferencia del OA por poseer un grupo hidroxilo en el carbono 2, mientras que en el OA está ausente. El UV y el ER comparten la misma estructura, excepto por la localización de un grupo metilo en carbonos diferentes.

Podemos encontrar estos compuestos en las hojas de *Olea europaea*, árbol comúnmente conocido como olivo. También existe una fracción triterpénica en el fruto del olivo, la aceituna y por tanto en sus productos, la aceituna de mesa y el aceite de oliva.

En las distintas variedades de aceituna de mesa (desde las verdes hasta las negras) el contenido en triterpenos oscila entre los 460 y los 1470 mg/kg. La pulpa de las aceitunas negras sin tratar contienen más de 2000 mg/kg. La hoja contiene importantes porcentajes de OA (3-3.5% del peso) seguido por el MA y en menores cantidades el UV y el ER.

En el aceite de oliva virgen la concentración de estos compuestos oscila entre 8.90 y 112.36 mg/kg. Estas diferencias se deben a factores genéticos (dependiendo de la variedad, el contenido en triterpenos es mayor) y a la manipulación y extracción del aceite. Las concentraciones de triterpenos son mucho mayores en aceites de orujo de oliva que en aceites de oliva vírgenes.

Hay pocos estudios que desvelen la biodisponibilidad de estos compuestos tras su consumo con los alimentos que los contienen, pero por ejemplo el OA tras la administración de 50 mg/kg a ratas, se absorbió mínimamente con una biodisponibilidad oral absoluta de 0,7%. En humanos, la concentración plasmática en la que aparece el OA tras una ingesta única oral de 40 mg fue de 26.5 ± 15 nM a las 5.2 ± 2.9 h. El MA se estudió en ratas tras una única administración de 50 mg/kg y se encontraron restos del compuesto 10 minutos después de la ingesta e incluso una hora después en sangre. Con UV y ER no existen, en la actualidad, estudios que puedan aportar datos sobre su biodisponibilidad.

Pero se debe dar especial importancia a las concentraciones en las que se puedan utilizar estos productos clínicamente en un futuro y estudiar en profundidad tanto el mecanismo de acción como los efectos secundarios que una administración diaria pudiera tener, pues dependiendo de la concentración utilizada podemos tener efectos bifásicos e incluso contrarios. Además de los efectos tóxicos que estos compuestos pueden llegar a tener en ratones y ratas, donde se ha visto que el MA no presenta efectos secundarios tras 28 días de administración de 50 mg/kg, pero el OA sí presenta efectos hepatotóxicos en ratones de una manera dosis dependiente; de hecho provocaba pérdida de peso, inflamación y apoptosis hepatocelular.

4.2 Enfermedad cardiovascular.

Una de las propiedades bioactivas que presentan estos compuestos es su capacidad de prevención ante la aparición de enfermedades cardiovasculares. Tanto el OA, como el MA y el UV protegen ante la oxidación de la LDL, y poseen actividad cardioprotectora, siendo antiaterogénicos y antihipertensivos. El ER promueve la vasorelajación de los anillos aórticos y promueve una reducción de la hipertrofia cardiaca en ratones tras su administración diaria.

4.3 Cáncer.

Se han estudiado los efectos de estos compuestos en distintos tipos de células de origen tumoral y en casi todas ellas los triterpenos ejercen una actividad protectora ante el tumor e incluso tienen actividad antitumoral. De hecho tienen efectos en factores cruciales para el desarrollo del cáncer, como puede ser la angiogénesis o la metástasis.

Tanto el OA como el MA parecen poseer efectos antiangiogénicos afectando a la invasión y migración celular e incluso decreciendo los niveles de VEGF en células de cáncer de hígado. Tanto es así que actualmente los estudios se centran en análogos de estas moléculas como potentes fármacos antitumorales.

El OA también es capaz de inducir apoptosis mediante la activación de la vía intrínseca o mitocondrial en células de cáncer pancreático y carcinoma hepatocelular.

Además se ha observado que inhibe la proliferación y la formación de colonias, induciendo apoptosis mediante la vía de señalización mTOR en células de osteosarcoma. En células de melanoma y de cáncer de páncreas se ha observado que el tratamiento con OA es capaz de promover apoptosis, induciendo la p53, disminuyendo la expresión de Bcl-2 e incrementando la expresión génica de la proteína proapotótica Bax.

El MA es quimiopreventivo en células de cáncer de colon e incluso en ratones. Posee actividad antimetastásica en células de cáncer de próstata e induce apoptosis mediante la activación de la caspasa 3 *in vitro,* también inhibe la expresión de COX-2, así como la de NFκβ *in vitro.*

El dialcohol UV es capaz de promover apoptosis mediante la activación de las JNKs en células de astrocitoma, mientras que también promueve apoptosis mediante EROs en células de cáncer de mama.

El ER posee actividad como apoptótico en células de colon, mama y astrocitoma, así como efecto antiproliferativo en células de cáncer tanto humano como murino.

4.4 Inflamación, estrés oxidativo y daño oxidativo al ADN.

La inflamación está relacionada con múltiples enfermedades, como por ejemplo el cáncer. Actualmente se conoce que antes del proceso tumoral, las células del tejido suelen sufrir un proceso inflamatorio previo y en ese nicho es donde se puede desarrollar el tumor.

Así, se atribuyen al aceite de oliva virgen múltiples cualidades como antiinflamatorio en determinadas patologías, pero no se tiene del todo claro qué productos son los que logran paliar esta inflamación o si es el conjunto de ellos lo que lo logra. Por ello actualmente encontramos varios estudios que están investigando el grupo de compuestos o el compuesto que provea al aceite de oliva virgen de esta actividad antiinflamatoria. Entre los posibles, se estudian tanto la fracción triterpénica del aceite, como los triterpenos aislados que contiene el aceite de oliva.

El OA tiene propiedades antiinflamatorias tanto *in vitro* como *in vivo*. Inhibe la activación de NFκβ en el núcleo y la producción de TNF-α en células endoteliales humanas de la vena umbilical. Varios estudios apuntan a que la activación de una manera eficiente de NFκβ mediante la producción de TNF-α está directamente relacionada con el estado redox de la célula; de manera que aunque se activara TNF-α, si la célula no se encuentra en un equilibrio redox óptimo, la cascada proinflamatoria no se activaría. Por tanto parece ser que el mecanismo redox celular y la inflamación están directamente relacionados, y que el OA podría ejercer su función antinflamatoria mediante tal mecanismo.

Químicamente estos compuestos parecen no ser muy eficientes como captadores de radicales libres, de hecho el MA es el único que a concentraciones muy altas (800 µM) posee una actividad débil como captador de radicales, aunque sí es muy efectivo como captador de radicales peroxilo en el ensayo ORAC.

Sin embargo, tanto OA como MA parecen prevenir ante la peroxidación lipídica de los microsomas en ratas *in vitro*. Efecto que se corroboró *in vivo* tras alimentar a los animales con aceite de orujo de oliva y observar que tanto el OA como el ER eran responsables, al menos en parte, de la prevención ante la peroxidación lipídica microsomal.

La inflamación y el estrés oxidativo están relacionados estrechamente no solo por la activación de la NFκβ, sino mediante otras vías antioxidantes como pueden ser la enzima COX (cyclo-oxygenase; ciclooxigenasa) o la 5-lipooxigenasa (5-LOX), ambas inhibidas tras tratar con OA. Además el MA, aparte de impedir la translocación al núcleo de la NFκβ, también promueve la inhibición de la COX-2 y la iNOS a nivel de ARNm (ácido ribonucleico mensajero), señales que activan la cascada proinflamatoria mediante la expresión de las citoquinas IL-6, IL-1β y TNF-α.

Además el OA promueve el estrés oxidativo en las células PC12, mediante el ahorro de glutatión (GSH), incrementando la actividad de la SOD y la CAT y reduciendo la liberación de IL-6 y TNF-α. OA también es neuroprotector al disminuir las EROs de manera dosis dependiente en el cerebro de ratón.

Asimismo estos compuestos protegen ante el daño oxidativo al ADN en células de leucemia y cáncer de mama. Aunque hacen falta más estudios para corroborarlo, podrían ser efectivos ante varias enfermedades relacionadas con el estrés oxidativo, como pueden ser el cáncer, enfermedades cardiovasculares, enfermedad de Parkinson o incluso Alzheimer. Aparte podrían influir en el envejecimiento, ya que la mayoría de enzimas que padecen este proceso sufren modificaciones oxidativas que producen su acumulación en los tejidos.

Aunque sobre el efecto antioxidante de estos compuestos hay relativamente pocos estudios, parece ser que es éste el mecanismo principal que utilizan, y podrían ser ellos los que en parte proporcionan propiedades bioactivas al aceite de oliva virgen. Pero son necesarios más estudios que permitan esclarecer los mecanismos de acción que utilizan estos compuestos y la biodisponibilidad real que las células podrían tener tras el consumo de estos compuestos mediante la dieta.

DISCUSIÓN

Discusión de los resultados

   1. Trabajo experimental titulado "Oleanolic acid selectivity inhibits proliferation of highly invasive human breast cancer cells and protects against oxidative DNA damage in normal mammary epithelial cells".

   1.1 Acción antitumoral de los compuestos ácido oleanólico y ácido maslínico en la línea tumoral humana de mama MDA-MB-231.

Varios estudios indican los efectos antitumorales de los triterpenos en distintos tipos de cáncer (Sánchez-Quesada *et al.* 2013), pero hasta ahora este es el primer estudio donde se investigan los efectos de los ácidos oleanólico (OA) y maslínico (MA) tanto en una línea tumoral altamente invasiva (MDA-MB-231) como en una línea epitelial de mama (MCF10A).

Estudios realizados en células de cáncer de mama ER- (estrógeno receptor negativas) demuestran que el OA es capaz de producir apoptosis (Chu *et al.* 2010). Cabe destacar que las pacientes con un genotipo ER- son consideradas pacientes con un mayor riesgo de desarrollar una metástasis más agresiva que aquellas que poseen el genotipo ER+ (Sheikh *et al.* 1994). Resultados anteriores obtenidos por nuestro grupo apuntan a que también en células tumorales ER+ (como la línea MCF7), el OA produce apoptosis (Allouche *et al.* 2011) y en el presente estudio se describe la acción citotóxica en las células de la línea tumoral ER- (MDA-MB-231), al igual que en el estudio de Chakravarti, el OA produce apoptosis también en las células de esta línea tumoral (Chakravarti *et al.* 2012) con lo que parece ser que el receptor de estrógenos es independiente del efecto citotóxico de estos compuestos.

Se conoce la relación directa entre estrés oxidativo en la célula y la muerte celular por apoptosis (Bonnet *et al.* 2007), de hecho tanto la vía de apoptosis intrínseca (o mitocondrial) como la vía extrínseca (o mediada por los receptores de la muerte celular) parecen relacionarse entre sí para promover la apoptosis dentro de la célula siendo los niveles de EROs responsables, entre otros factores, de esta interrelación. En la vía apoptótica extrínseca actúan moléculas relacionadas con el receptor del factor de necrosis tumoral (TNFR; tumor necrosis factor receptor), activando una cascada de señalización que activa la procaspasa-8. Por otro lado, la vía intrínseca se activa tras estímulos intracelulares que inician eventos dentro de la célula. Estos eventos provocan modificaciones del potencial de membrana de la mitocondria que producen un cambio en la permeabilidad, pérdida del potencial transmembrana mitocondrial y la liberación de proteínas pro-apoptóticas del espacio intermembranoso al citosol. Entre estas proteínas apoptóticas se encuentra el

citocromo c, entre otros. Este citocromo c, que en condiciones normales funciona como transportador electrónico mitocondrial, pasa al citosol donde se une y activa a la proteína Apaf-1 (Apoptosis protease-activating factor-1), la cual desencadena la formación del apoptosoma, y la activación de la procaspasa-9. Ambas vías se relacionan por ejemplo mediante la vía apoptótica Fas, mediada por la escisión de la caspasa-8 de la proteína Bid (Mukhtar *et al.* 2012). Entre los eventos intracelulares que provocan la activación de la vía apoptótica mitocondrial se encuentra la producción de EROs mitocondrial. Así, la misma producción de EROs mitocondrial influenciaría en la fosforilación de determinadas proteínas reguladoras del proceso de apoptosis intrínseca. E incluso podrían tener un papel en la activación de la vía extrínseca de la apoptosis, ya que varios estudios realizados con antioxidantes demuestran que éstos son capaces de bloquear la vía inducida por las proteínas de la familia TNF (Simon *et al.* 2000).

Los efectos observados en MDA-MB-231 tras el tratamiento con OA en el estudio de estrés oxidativo, podrían estar relacionados con la muerte celular observada en proliferación. La vía intrínseca de la apoptosis está regulada por un conjunto de proteínas de la familia Bcl-2 que controlan la permeabilidad de la membrana mitocondrial y regulan la liberación del citocromo c. Esta familia de proteínas está compuesta por proteínas proapoptóticas (Bcl-10, Bax, Bak, Bid, Bad,Bim, Bik y Blk) y proteínas antiapoptóticas (Bcl-2, Bcl-x, Bcl-xL, Bcl-xS, Bcl-w, BAG) y regulada por el gen supresor de tumores p53. Así, un cambio de expresión, por ejemplo, de Bcl-2 y de Bax, podría explicar la reducción de la proliferación celular observada (Mukhtar *et al.* 2012). Pero el estudio sobre apoptosis no mostró cambios algunos en la población que demostraran que se producía tal apoptosis, por tanto los mecanismos mediante los que las EROs pueden estar produciendo una reducción de la proliferación no están del todo claros, aunque todo indica que podrían estar implicadas en este proceso. De hecho otros estudios sí que apuntan a que el OA es capaz de producir apoptosis en células tumorales de la línea MDA-MB-231, así como detener el ciclo celular de éstas (Chakravarti *et al.* 2012).

Asimismo, el OA promueve apoptosis mediante la vía intrínseca en células de carcinoma hepatocelular, de cáncer pancreático, en células de melanoma y de cáncer de colon (Sánchez-Quesada *et al.* 2013).

Además, los resultados obtenidos en el análisis del daño al ADN celular muestran que el OA es capaz de promover daño al ADN de la línea tumoral de mama altamente invasiva, MDA-MB-231. Se conoce que las EROs pueden producir daños en el ADN, tales como mutaciones, roturas de hebra de la doble cadena de ADN, intercambio de cromátidas hermanas, oxidación específica de pirimidinas y purinas o incluso, la formación de micronúcleos (Lopaczynski y Zeisel 2001).

Así el OA es capaz de alterar los niveles de EROs intracelulares, tener un efecto antiproliferativo y promover daño al ADN. Todos estos efectos antitumorales podrían estar conectados entre sí mediante mecanismos de alteración del estrés oxidativo, lo que implicaría que mediante la regulación de éste, el OA podría ser capaz de promover la muerte de la célula tumoral de mama. Un elevado nivel de EROs es una característica intrínseca de las células tumorales en comparación con una célula normal, al igual que el aumento de los niveles de EROs por encima del umbral homeostático crítico es incompatible con el crecimiento o la supervivencia de las células tumorales, mientras que puede ser tolerado por las células normales. De hecho en clínica se usan agentes genotóxicos, como el cisplatino, dependientes de la producción de EROs para su eficacia. Aunque uno de los efectos secundarios más acusados que produce el incremento de EROs en tejido normal, es la función defectuosa de la respuesta inmune, concretamente en dos tipos celulares los macrófagos y los neutrófilos, donde las EROs ya se encuentran aumentadas intrínsecamente para desempeñar papeles de señalización fundamentales. Además no todos los tipos celulares tienen la misma sensibilidad a la producción de EROs, siendo más resistentes las células de origen epitelial que las de origen mesenquimal (Boland *et al.* 2013).

Los efectos observados en el caso del MA en la línea tumoral de mama MDA-MB-231, no son muy acusados. Aunque parece tener efectos antiproliferativos en estas células a las 48 y 72 h, parecen no estar relacionados con el estrés oxidativo, al menos en un principio, ya que éste no se presentaba alterado. Sin embargo, una vez que se indujo un daño oxidativo al ADN celular, el MA parecía potenciar esa actividad, produciendo mayor daño aún. Aunque no se apreciaron efectos de apoptosis ni variaciones en el ciclo celular, se necesitarían más ensayos para poder asegurar que este compuesto no posee ninguna actividad apoptótica en la línea altamente invasiva de mama MDA-MB-231. De hecho este compuesto es uno de los cuatro triterpenos con mayor efecto antitumoral y antimetastásico en otros tipos de tumores sólidos, incluso promueve apoptosis mediante la ruta de las caspasas (Sánchez-Quesada *et al.* 2013).

1.2 Acción preventiva de los compuestos ácido oleanólico y ácido maslínico en la línea humana epitelial de mama MCF10A.

Ambos compuestos son citotóxicos, antiproliferativos y proapoptóticos en la línea normal epitelial de mama MCF10A a altas concentraciones. Pero a bajas, no sólo no dañan a la célula, sino que incluso promueven el crecimiento celular.

La acción que producen ambos compuestos en los niveles de estrés oxidativo intracelulares en la célula no tumoral nunca antes se ha descrito. El OA parece proteger a la célula ante el estrés oxidativo, efecto contrapuesto al MA, que

incrementa incluso la cantidad de EROs intracelulares, una vez que se ha inducido un choque oxidativo. Este resultado se correlaciona con los niveles de CAT activa de la célula, que aparecen más elevados que en el control.

Pero el efecto más relevante del OA es su actividad protectora ante el daño oxidativo al ADN (acción que no produce el MA). Por ello este compuesto podría utilizarse como quimiopreventivo en cáncer de mama. Esta actividad podría estar relacionada directamente con la capacidad de este compuesto para controlar los niveles de EROs intracelulares. Así, se ha descrito que igual que las EROs son capaces de promover apoptosis e incluso necrosis en células tumorales, podrían activar vías antiapoptóticas (dependiendo del nivel en el que se hallen) en la célula, mediante la activación de factores de transcripción como el NF-κβ (Simon *et al.* 2000).

En la actualidad no hay trabajos que señalen las posibles vías de acción preventiva de estos compuestos en líneas no tumorales de mama, pero sí hay estudios que demuestran su capacidad preventiva en células musculares lisas del tejido vascular o ante el daño cardiaco en ratones con miocarditis autoinmune, dónde se ven afectadas vías relacionadas con el estrés oxidativo (Martín *et al.* 2014; Qin *et al.* 2014). Por ello parece que estos compuestos deben sus efectos a la acción que ejercen en el estrés oxidativo intracelular, mediante la activación o el reforzamiento quizás de enzimas antioxidantes (de hecho en este trabajo la actividad de la CAT parece aumentar con la concentración de OA) o por la activación de vías como la Akt/NRF2/HO-1, implicadas en el metabolismo antioxidante celular. Mediante la vía del NRF2 (nuclear respiratory factor 2) se promueve la biogénesis mitocondrial, metabolismo celular y respuestas antioxidantes mediante la coordinación de factores de transcripción como el ERRα (estrogen related receptor alpha) y PPARγ (peroxisome-proliferator activator receptor-gamma) (Boland *et al.* 2013).

2. Trabajo experimental titulado "The differential localization of a methyl group confers to two triterpenes present in the olives a different anti-breast cancer activity".

2.1 Acción antitumoral de los compuestos uvaol y eritrodiol en la línea tumoral humana de mama MDA-MB-231.

Según los resultados obtenidos UV solo posee actividad antitumoral en esta línea altamente invasiva de mama a la concentración mayor. Concentraciones menores provocan un efecto antioxidante en la célula y protegen ante el daño oxidativo inducido al ADN. Sin embargo ER difiere en su acción, provoca un efecto antioxidante intracelular pero causa daño al ADN. Esta disparidad en los efectos que ambos

provocan podría ser debida a la molécula de CH_3 localizada en carbonos diferentes en los dos compuestos.

Otros estudios presentan los efectos apoptóticos que poseen estos compuestos en la línea tumoral de mama MCF7 (Allouche *et al.* 2011) y en una línea de astrocitoma mediante la activación de quinasas c-Jun N-terminal (JNKs) (Sánchez-Quesada *et al.* 2013), asociadas a los niveles de EROs intracelulares. Aunque los estudios con estos compuestos en cáncer son limitados, otro trabajo más apunta la acción apoptótica del ER en células de cáncer de colon (HT29) mediante el aumento de actividad de la caspasa 3 (Juan *et al.* 2008).

Ambos compuestos alteran el equilibrio oxidativo en esta línea tumoral, pero debido a la falta de estudios que corroboren qué vías de señalización se están alterando, no se puede discernir si el efecto antioxidante es una consecuencia de la alteración de estas vías o la causa por la que se altera el equilibrio oxidativo de la célula cancerígena. Al alterar estos niveles intracelulares de EROs, puede ser éste, como hemos visto anteriormente, el encargado de la expresión o inhibición de determinados tipos de genes. Las EROs regulan la activación y duración de vías de transducción de señales dependientes del equilibrio reducción-oxidación (redox) mediante reducciones/oxidaciones cíclicas de los residuos de cisteína de determinadas quinasas, fosfatasas y otros factores de regulación. Dependiendo de la concentración de H_2O_2 existente, tendremos diferentes oxidaciones de cisteínas en la vía de señalización, y por tanto, respuestas contrarias en la proliferación celular, senescencia o incluso muerte celular.

Aunque los mecanismos de acción no están del todo claros aún, si parece ser que ambos compuestos poseen actividades distintas en esta línea, y la única diferencia que poseen es la localización, en carbonos diferentes, de un grupo metilo.

2.2 Acción preventiva de los compuestos uvaol y eritrodiol en la línea humana epitelial de mama MCF10A

A pesar del efecto tóxico que poseen en la línea epitelial de mama MCF10A a altas concentraciones, ambos compuestos son capaces de prevenir el estrés oxidativo intracelular tanto en condiciones basales, como tras un choque oxidativo.

De hecho UV presenta un efecto muy acusado de prevención de roturas en las hebras de ADN de la célula en condiciones normales, por lo que este compuesto podría actuar en la prevención del daño al ADN en las células epiteliales de mama, previniendo así ante la aparición de cáncer. Pero una vez que la célula ha estado expuesta a unos niveles altos de EROs, el UV pierde el efecto de protección, incrementando incluso el daño al ADN.

Se sabe que las EROs están implicadas en la regulación redox de la célula, regulando la expresión génica, los daños oxidativos a la célula y la actividad citotóxica del sistema inmune. En cáncer las EROs pueden causar daños permanentes al ADN, causando por ejemplo mutaciones en el gen supresor de tumores, p53, frecuentemente mutado en células tumorales en más del 50% de los casos (Saeidnia y Abdollahi 2013). La proteína supresora de tumores, p53 es un factor de transcripción encargado de la entrada de la célula en el ciclo celular y posee una conexión sinérgica que ejemplifica la extrema complejidad de la regulación redox del ciclo celular. La sobreexpresión de p53 induce la activación de varias enzimas pro-oxidantes como la prolina oxidasa y genes como el p66 que interactúan con el citocromo c para incrementar los niveles de H_2O_2 en la mitocondria. Pero hay evidencias que indican que la activación redox de p53 implica modificaciones postraduccionales como la modulación redox de residuos de cisteína críticos en sus dominios de unión al DNA. De manera que cuando existe una producción equilibrada de EROs y p53 en células normales, puede ser interrumpida por un incremento pequeño de p53 que incrementa discretamente los niveles de EROs, lo que a su misma vez, produce la activación de quinasas proliferativas como Akt (protein kinase B; proteína quinasa B) y ERK1/2 (extracellular signal-regulated kinase; quinasa reguladora por señales extracelulares), dando lugar a una proliferación exacerbada o incluso a hipertiroidismo. Una alta concentración de p53 detiene el ciclo celular y produce apoptosis, junto con un descenso de Akt, incremento de la actividad respiratoria y aumento dramático de los niveles de EROs. Por otro lado, un descenso de p53, reduce la actividad de la citocromo c oxidasa, disminuye las EROs y promueve el nicho para la transformación celular (por ejemplo para la carcinogénesis) (Antico Arciuch *et al.* 2012). Así un pequeño aumento de los niveles de EROs puede promover la proliferación, pero en cuanto ese aumento es crítico, las EROs producen un aumento de la p53 que desencadena en muerte celular, esto podría explicar los efectos que observamos con el ER, que detiene el ciclo y promueve apoptosis a determinadas concentraciones. De hecho la vía de señalización JNK, relacionada con la regulación apoptótica celular (donde interviene la p53) y con el estrés oxidativo en varias líneas celulares, ha sido estudiada y se ha visto que se activa tras el tratamiento con este compuesto en diferentes líneas tumorales (Martín *et al.* 2009).

Por el contrario, el efecto de UV en las células puede prevenir estas mutaciones y así la aparición del cáncer, porque previene tanto el aumento de los niveles intracelulares de EROs, como el daño al ADN. Pero cabe destacar, que la proliferación celular depende del equilibrio entre el nivel de EROs y los antioxidantes presentes. Así vemos que este compuesto, a baja concentración, parece actuar como antioxidante para las células normales de mama *in vitro*, pero a altas concentraciones tiene efectos antiproliferativos.

ER provoca justo el efecto contrario, incrementando el daño al ADN tanto en condiciones normales como en condiciones de exposición a H_2O_2. Aunque se comporta como un antioxidante, provoca apoptosis y detiene el ciclo celular.

3. Trabajo experimental titulado "Maslinic acid improves natural immune response through inhibition of chronic inflammation".

Una vez que la línea celular THP-1 fue diferenciada a macrófago y estimulada para su polarización a macrófago tipo M1, se pasó a estudiar los efectos que producían los cuatro triterpenos en la producción de citoquinas relacionadas con la inflamación.

Los macrófagos que forman parte de la masa tumoral, llamados TAMs (tumor associated macrophages) pueden estar polarizados como M1 o M2. Los TAMs polarizados a M1 (o clásicamente activados) se caracterizan por su actividad proinflamatoria, por la presentación de antígenos y por poseer la capacidad de lisar tumores. Los TAMs de tipo M2 (o alternativamente activados) poseen baja capacidad presentadora de antígenos, una producción alta de citoquinas antiinflamatorias, median la angiogénesis y promueven la cicatrización de heridas (Laoui *et al.* 2011; Mantovani y Sica 2010).

En carcinoma pulmonar el hecho de tener un alto índice en la relación M1/M2 incrementa la supervivencia (Heusinkveld y Van der Burg 2011). Además en tumores malignos los TAMs suelen presentar el fenotipo M2 (Mantovani y Sica 2010; Qian y Pollard 2010).

Parece ser que los macrófagos M1 actuarían en las etapas tempranas del desarrollo del tumor produciendo citoquinas proinflamatorias responsables de activar la respuesta inmunitaria y en teoría prevenir el crecimiento tumoral. Pero parece que en el proceso, ya sea por las citoquinas que produce la masa tumoral o por señales externas, estos macrófagos pasan a tener un fenotipo M2 (Solinas *et al.* 2009). Según los resultados obtenidos, los macrófagos M1 tratados con MA parecen potenciar la respuesta antitumoral, pudiendo posiblemente, hacerla más eficaz en las primeras etapas del desarrollo del tumor e impidiendo el crecimiento de éste. Para ello incrementan las señales para el reclutamiento de más macrófagos en el sitio de la inflamación (mediante la producción de IL-8, IL-1α e IL-1β) consiguiendo un efecto llamada y haciendo que estos macrófagos se polaricen al tipo M1, ya que aumentan la señal de INF-γ e impiden la producción de señales como IL-10 (citoquina responsable de la polarización del macrófago a M2).

Además no modifican señales directas de la promoción de la inflamación crónica como lo son NFκβ o NO.

Discusión de los resultados

Estos efectos proinflamatorios se observaron a concentraciones bajas, los efectos a concentraciones mayores son de hecho citotóxicos para el macrófago. Por tanto a menores concentraciones el MA lograría promover una respuesta antitumoral por parte del sistema inmune evitando el desarrollo tumoral.

CONCLUSIONES FINALES

Conclusiones

1) Se ha demostrado el potencial efecto antitumoral del ácido oleanólico y ácido maslínico frente a células tumorales de mama humanas altamente invasivas.

2) Se ha demostrado que el ácido oleanólico y el uvaol protegen ante el daño oxidativo en las células epiteliales de mama humanas.

3) Tanto el ácido maslínico como el eritrodiol modulan la inflamación en un modelo experimental celular.

4) Los resultados obtenidos confirman que los compuestos triterpénicos estudiados poseen propiedades que podrían indicar que contribuyen a la acción protectora frente al desarrollo del cáncer de mama, asociada al consumo habitual de aceites de oliva vírgenes. No obstante, hay que tener en consideración que nuestros estudios se han realizado *in vitro* con modelos experimentales celulares y serían necesarios estudios adicionales para poder establecer esta aseveración.

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ANEXO

Otros trabajos publicados:

Warleta, F., Campos, M., Allouche, Y., Sánchez-Quesada, C., Ruiz-Mora, J., Beltran, G., and Gaforio, J. J. (2010). "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.,* 48(4), 1092-1100.

Revista: Food and Chemical Toxicology

Factor de Impacto: 2.610

Categoría: Tecnología y ciencias de la alimentación

Cuartil en la categoría: Q1

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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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.

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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 anti-proliferative capacity and oxidative DNA damage protection in both human breast cancer cell lines (MCF7 and MDA-MB-231)

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-methyl-phenazonium 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^{tm} (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:

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

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 (96°) 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:

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

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

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

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:

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

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:

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

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 (300g 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 (300g 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.

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

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
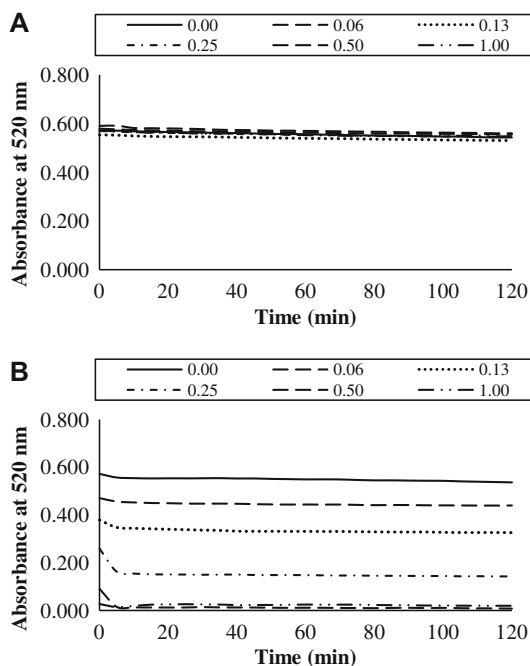
Fig. 1. Radical scavenging activity was measured by the reduction of the DPPH radical (100 µM) by ethanolic solutions of squalene (A) or α-tocopherol (TOC) (B) for a period of 120 min. The mole ratios [mole antioxidant/mole DPPH] assayed were between 0.06 and 1. The relative radical scavenging activity (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.
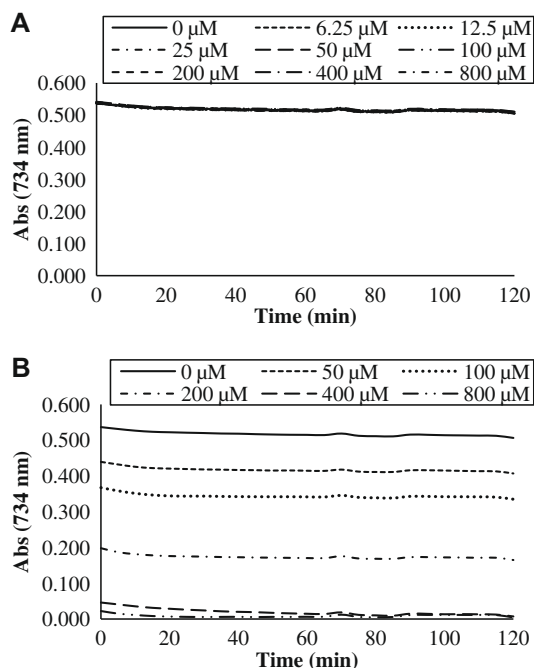
Fig. 2. ABTS radical cation (ABTS[·+]) antioxidant activity of ethanolic solutions of squalene (A) or Trolox™ (B) up to 800 µM were measured by decolorization of ABTS[·+] for 120 min. Relative radical scavenging activity (RSA) for all assayed concentrations of squalene at 30 min was <4% while the RSA of Trolox™ reached 50% at 143.57 µM.
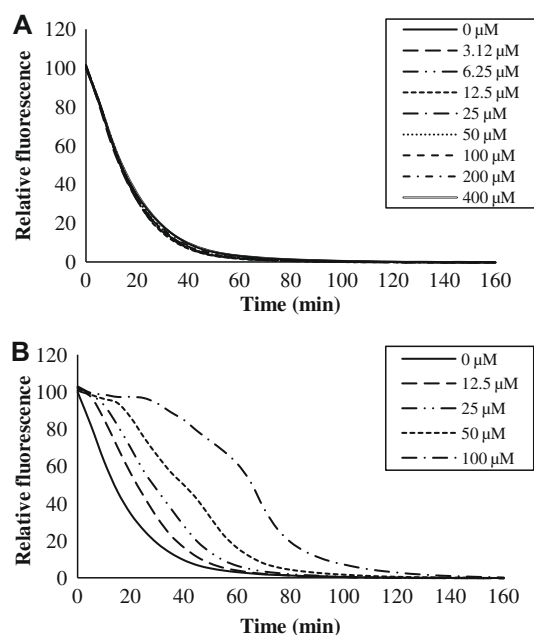
Fig. 3. Peroxyl antiradical activity was performed by the ORACFL assay on a range of concentrations of squalene from 3.12 to 400 μM (A) or Trolox™ from 12.5 to 100 μM (B) for 160 min. Results show how squalene failed to reduce the AAPH-induced peroxyl radical at all assayed concentrations compared with 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
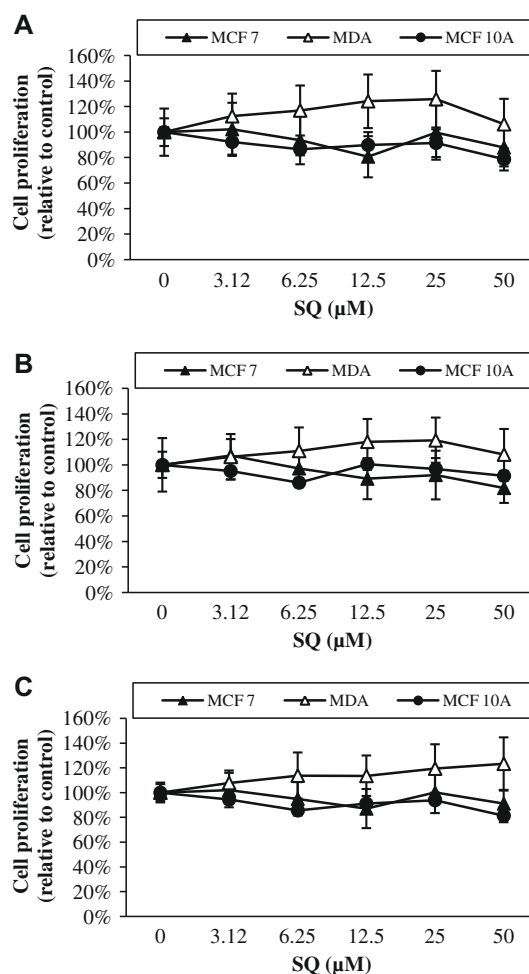
Fig. 4. *In vitro* cell proliferation of MCF7, MDAMB-231 and MCF10A cell lines after squalene (SQ) treatment was determined by colorimetric XTT-based assay at 24 h (A), 48 h (B) and 120 h (C). Although MDA-MB-231 showed a slight increase in cell proliferation, no difference at $p < 0.05$ was detected 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%.
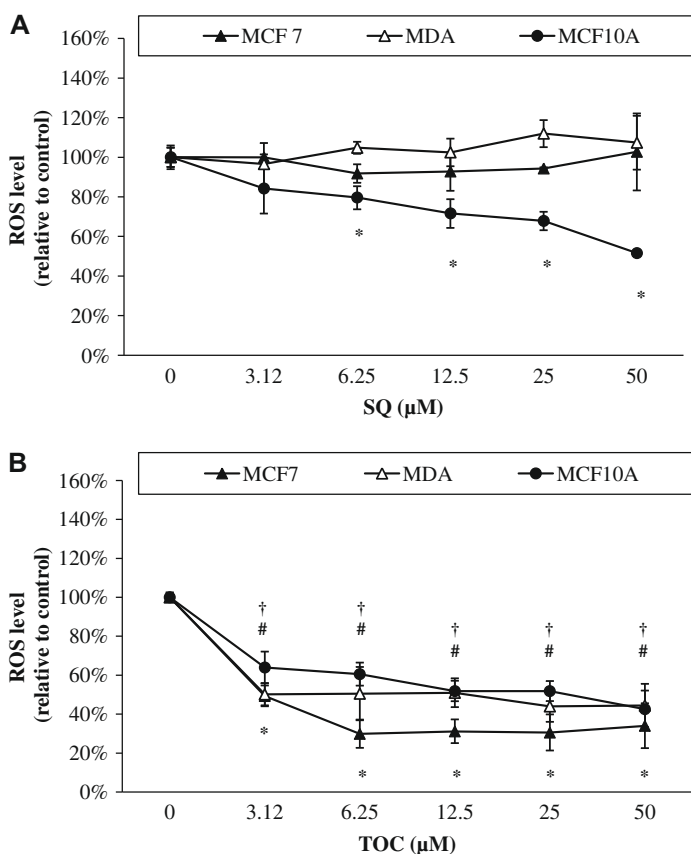
Fig. 5. Intracellular ROS levels were measured by DCFH-DA probe in MCF7, MDA-MB-231 and MCF10A cell lines after treatment with squalene (SQ) (A) or α-tocopherol (TOC) (B) for 24 h. Squalene significantly decreased intracellular ROS levels, in a dose-dependent manner, in MCF10A cells but not in MCF7 and 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
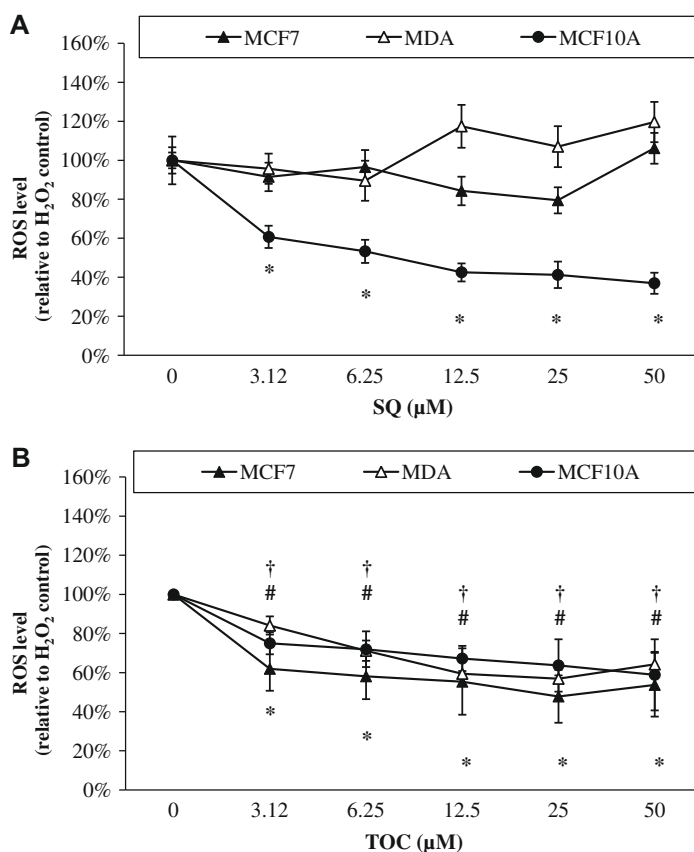
Fig. 6. The capacity of squalene (SQ) (A) or α-tocopherol (TOC), as antioxidant control (B), to reduce an oxidative burst induced by the addition of H_2O_2 (500 μM) on MCF7, MDA-MB-231 or MCF10A cell lines was measured by DCFH-DA probe. Squalene prevented the oxidative injury induced in MCF10A cells, 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
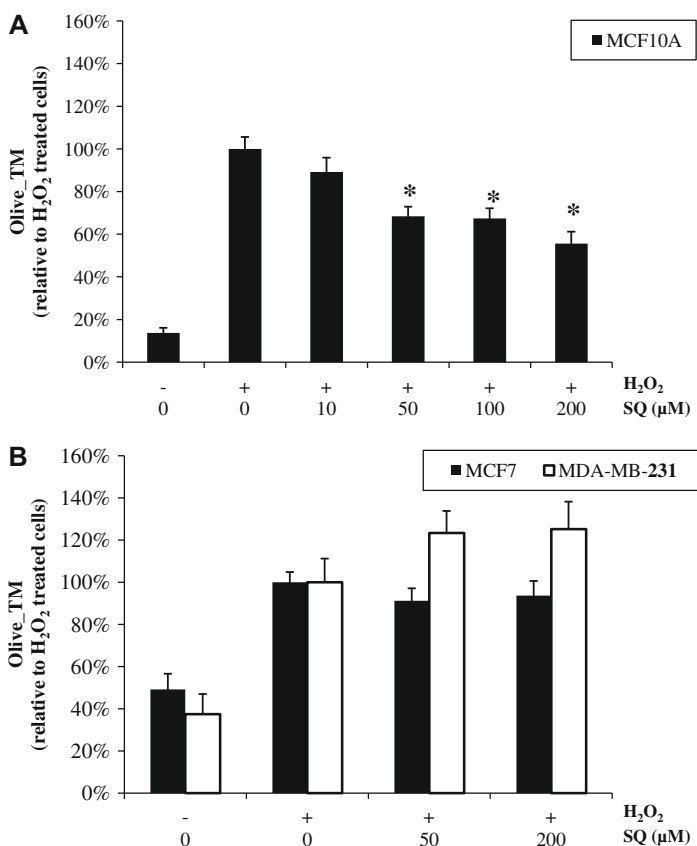
Fig. 7. A comet assay was performed in order to determine the preventive effect of squalene (SQ) in DNA oxidative damage in the MCF10 cell line (A) or MCF7 and MBA-MB-231 cell lines (B). Squalene reduces single-strand breaks, in a dose-dependent manner, in MCF10A cells, but not in MCF7 and MBA-MB-231 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,

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

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Allouche, Y., Warleta, F., Campos, M., Sánchez-Quesada, C., Uceda, M., Beltran, G., and Gaforio, J. J. (2011). "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." *J.Agric.Food Chem.,* 59(1), 121-130.

Revista: Journal of Agricultural and Food Chemistry

Factor de Impacto: 3.107

Categoría: Tecnología y ciencias de la alimentación

Cuartil en la categoría: Q1

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.

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

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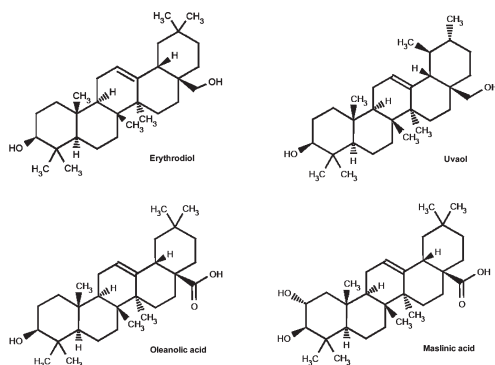
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Figure 1. Chemical structures of erythrodiol, uvaol, oleanolic acid, and maslinic acid.

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:

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

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 for 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[•+] (**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 were 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
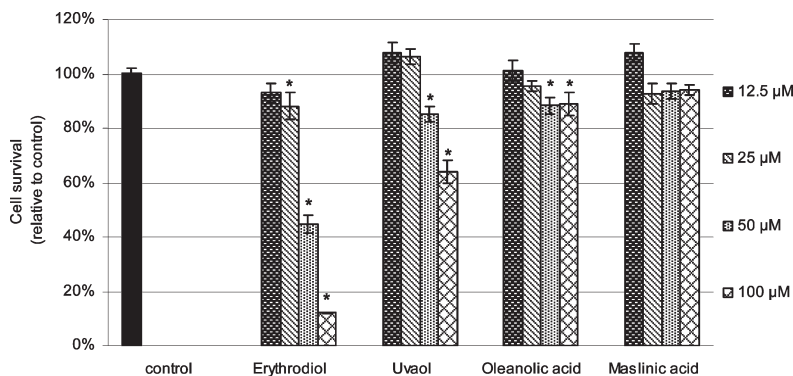
Figure 2. Effects of triterpenes on MCF-7 cell survival. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then cell survival was determined by the XTT assay. The results represent the mean ± SEM of three independent experiments. ∗ 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
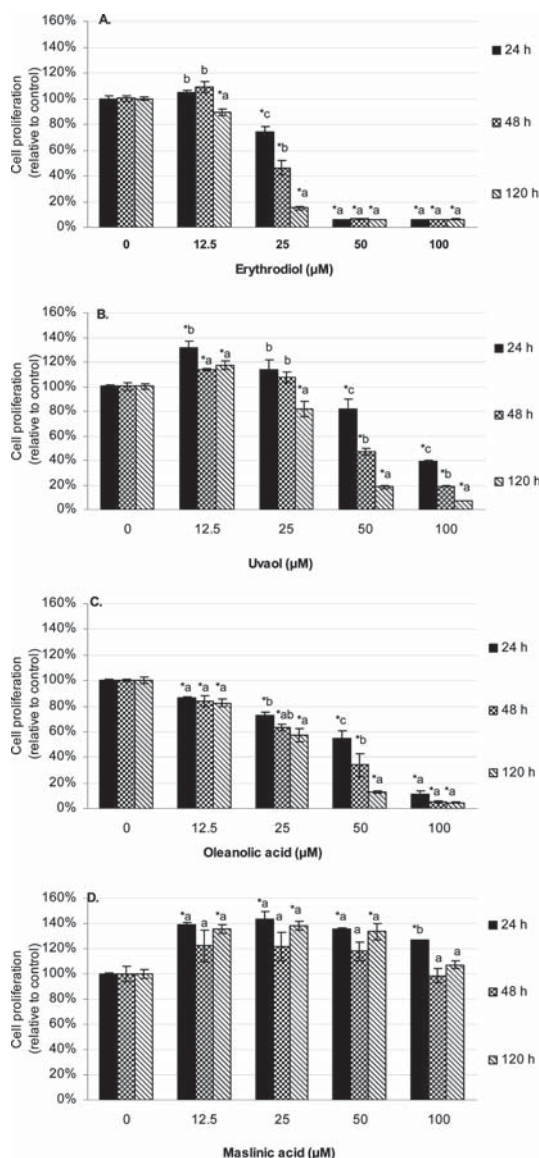
Figure 3. Effects of triterpenes on MCF-7 cell proliferation. Cells were treated with different concentrations of erythrodiol (**A**), uvaol (**B**), oleanolic acid (**C**), and maslinic acid (**D**) for 24, 48, and 120 h, and then cell proliferation, using the XTT assay, was determined after a proliferation 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
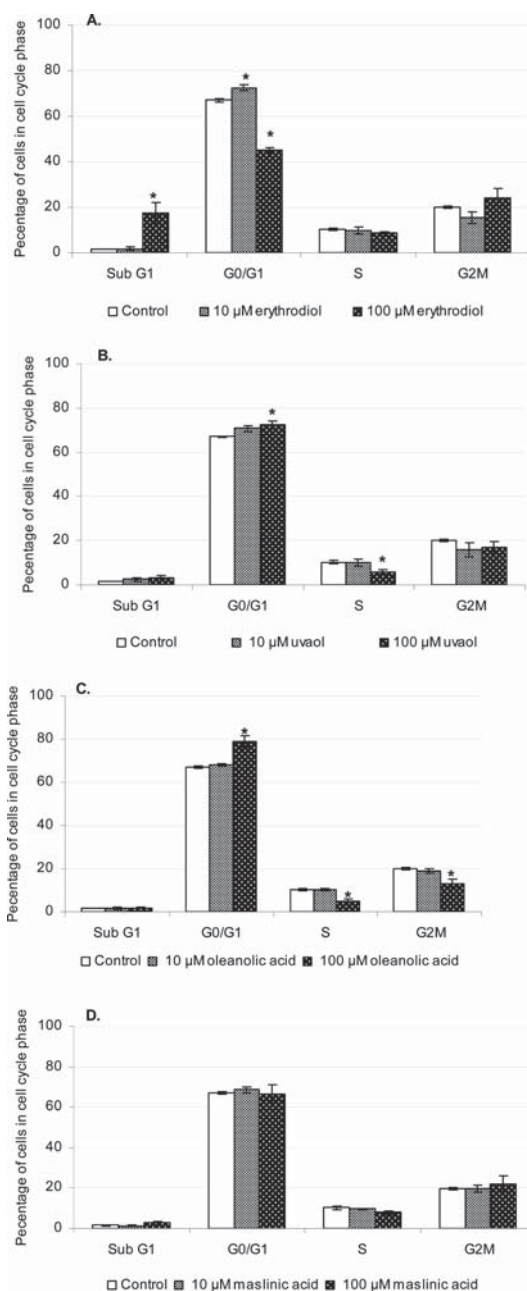
time-dependent manner, the effect of erythrodiol being more pronounced. Hence, these data suggest that these triterpenes effectively reduced the malignancy and suppressed the generation
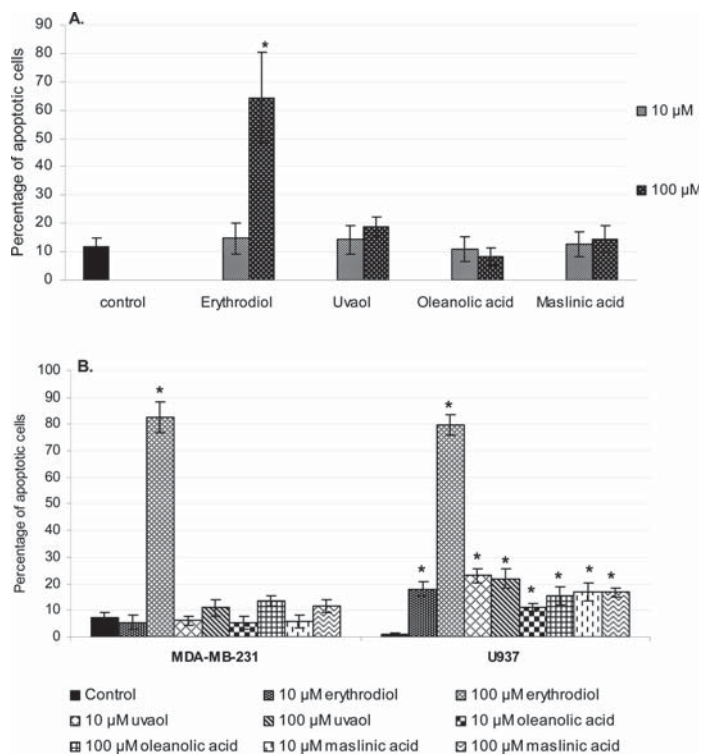
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Figure 5. Effects of triterpenes on apoptosis induction on MCF-7 (**A**) and MDA-MB-231 and U937 cells (**B**). Cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were collected and stained with FITC-conjugated 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.
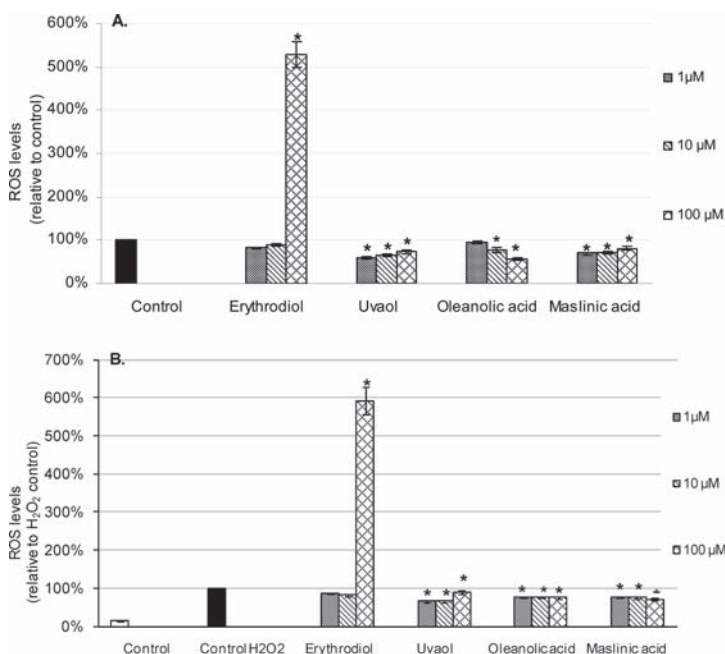
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Figure 6. (**A**) Intracellular ROS level in MCF-7 cells after triterpene treatment. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then intracellular ROS level was measured by 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.
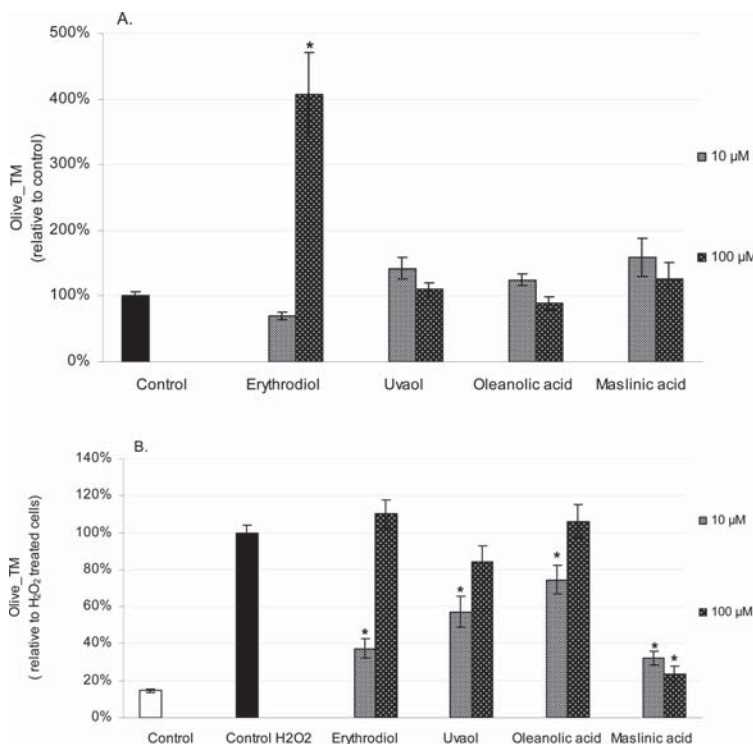
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Figure 7. (**A**) Effects of triterpenes on DNA integrity assessed by the comet assay. MCF-7 cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were subjected to comet assay. Olive_TM values represent the mean $\pm$ 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 $\pm$ 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

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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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Warleta, F., Sánchez-Quesada, C., Campos, M., Allouche, Y., Beltran, G., and Gaforio, J. J. (2011). "Hydroxytyrosol protects against oxidative DNA damage in human breast cells." Nutrients, 3(10), 839-857.

Revista: Nutrients

Factor de Impacto: 3.148

Categoría: Nutrición y dietética

Cuartil en la categoría: Q2

Article

Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells

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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.

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:

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

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:

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

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

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.

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

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.

(**a**)

(**b**)
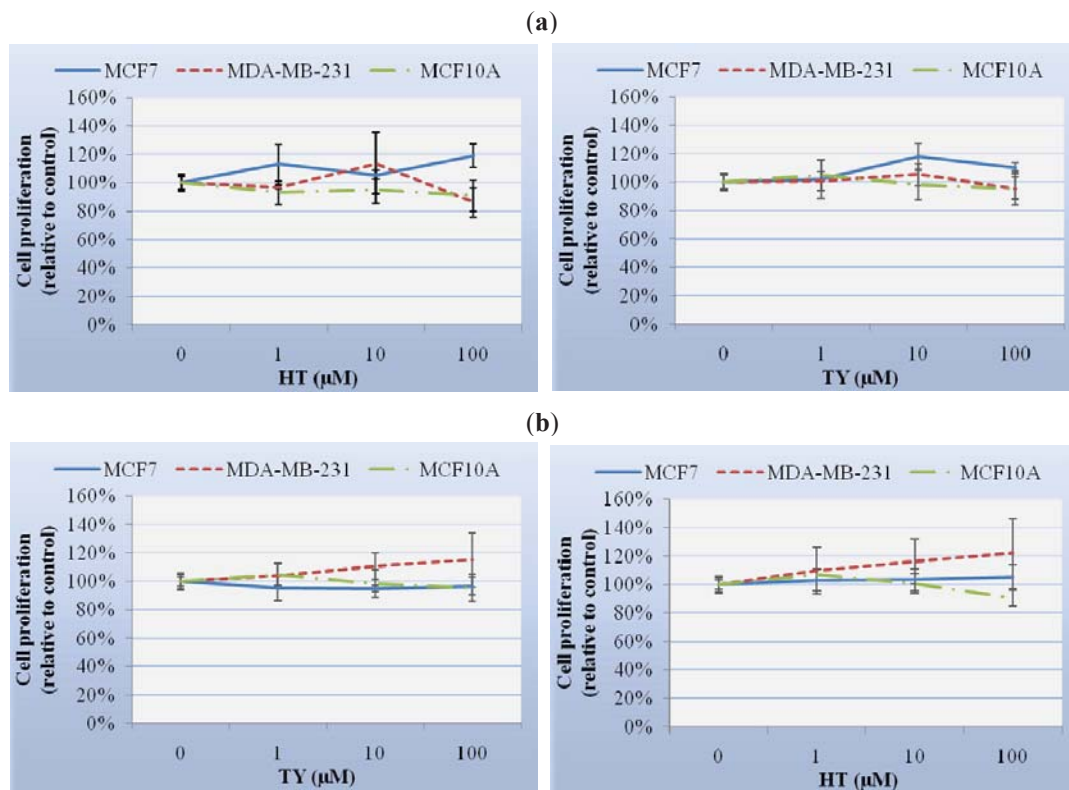
3.3. Cell Cycle and Apoptosis

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.
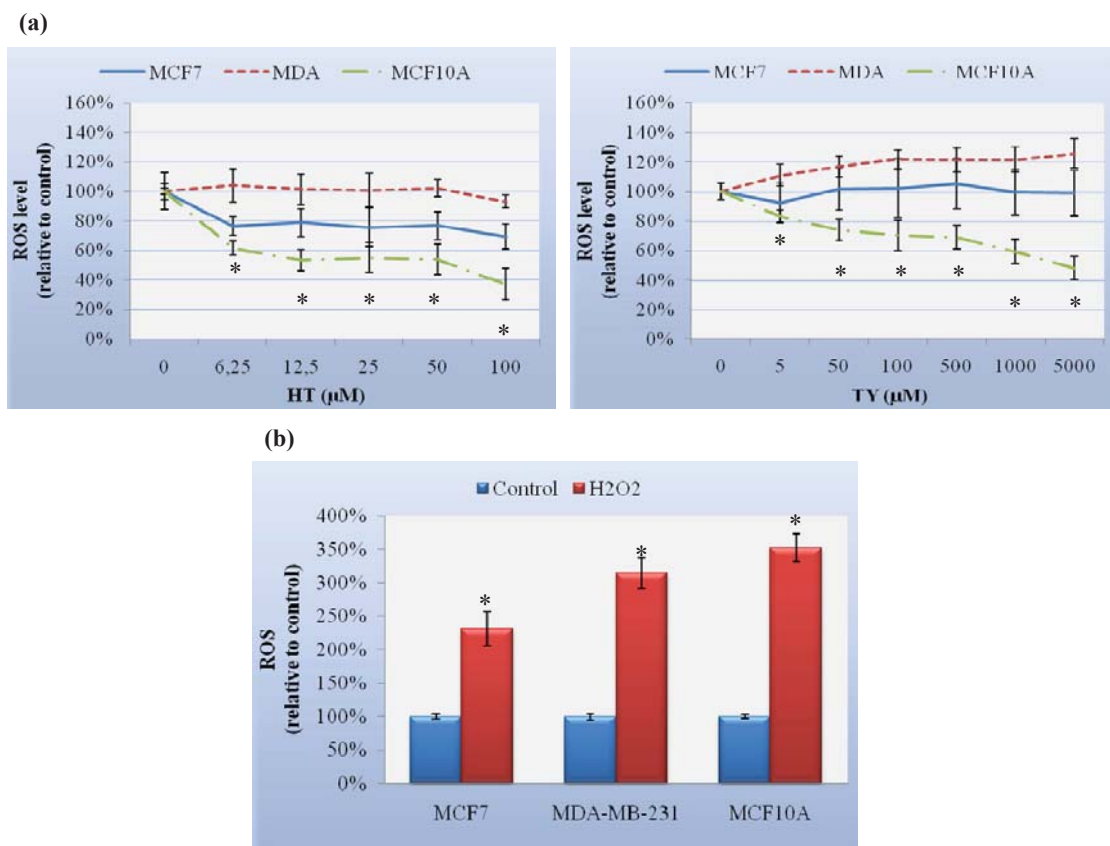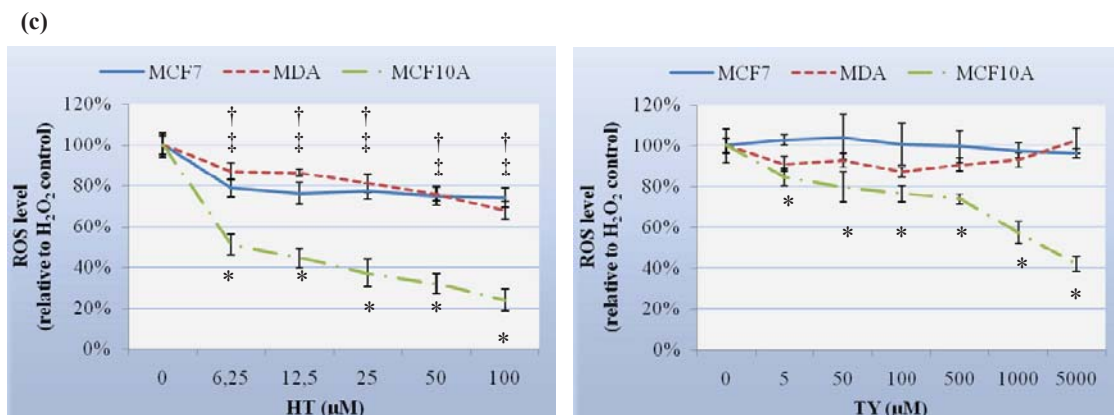
(a)

(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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ 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.
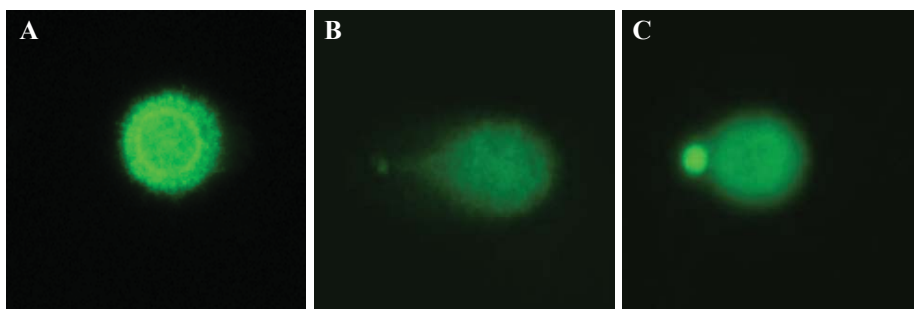
Breast cells exposed to H_2O_2 were effectively DNA damaged and the mean olive tail moment (Olive_TM) was determined by the Comet assay. Breast epithelial cells were the most sensitive to the H_2O_2-induced DNA damage (Figure 4(a)).

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)

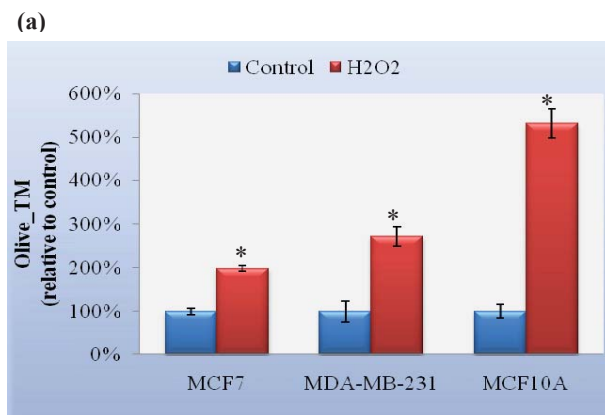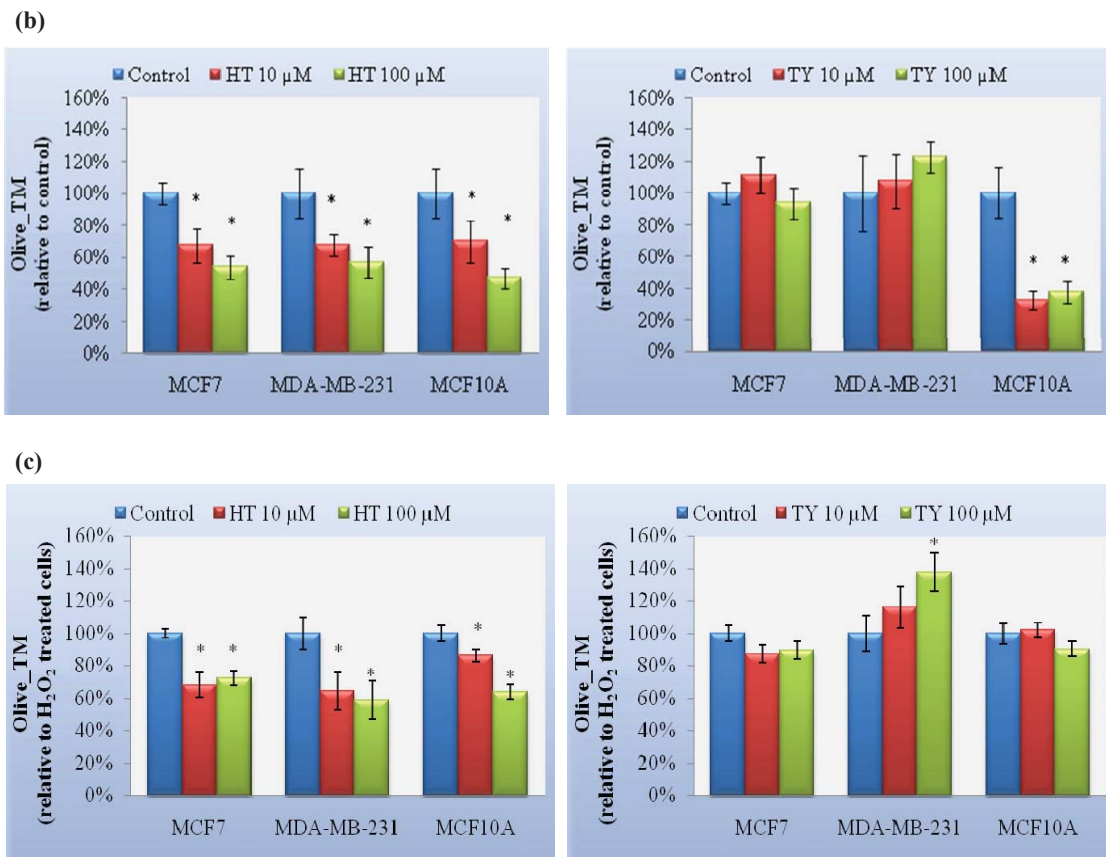## 4. Discussion

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

Conflict of Interest

The authors declare no conflict of interest.

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Campos, M., Luque, R.,Jiménez, J., Martínez, R., Warleta, F., Sánchez-Quesada, C., Delgado-Rodríguez, M., Calvo, A., Gaforio, J.J. (2013) "Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patients." Histol .Histopathol., 28, 1439-1450.

Revista: Histology and Histopathology

Factor de Impacto: 2.236

Categoría: Patología

Cuartil en la categoría: Q2

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

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 Immunohisto-chemistry 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 immuno-staining 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
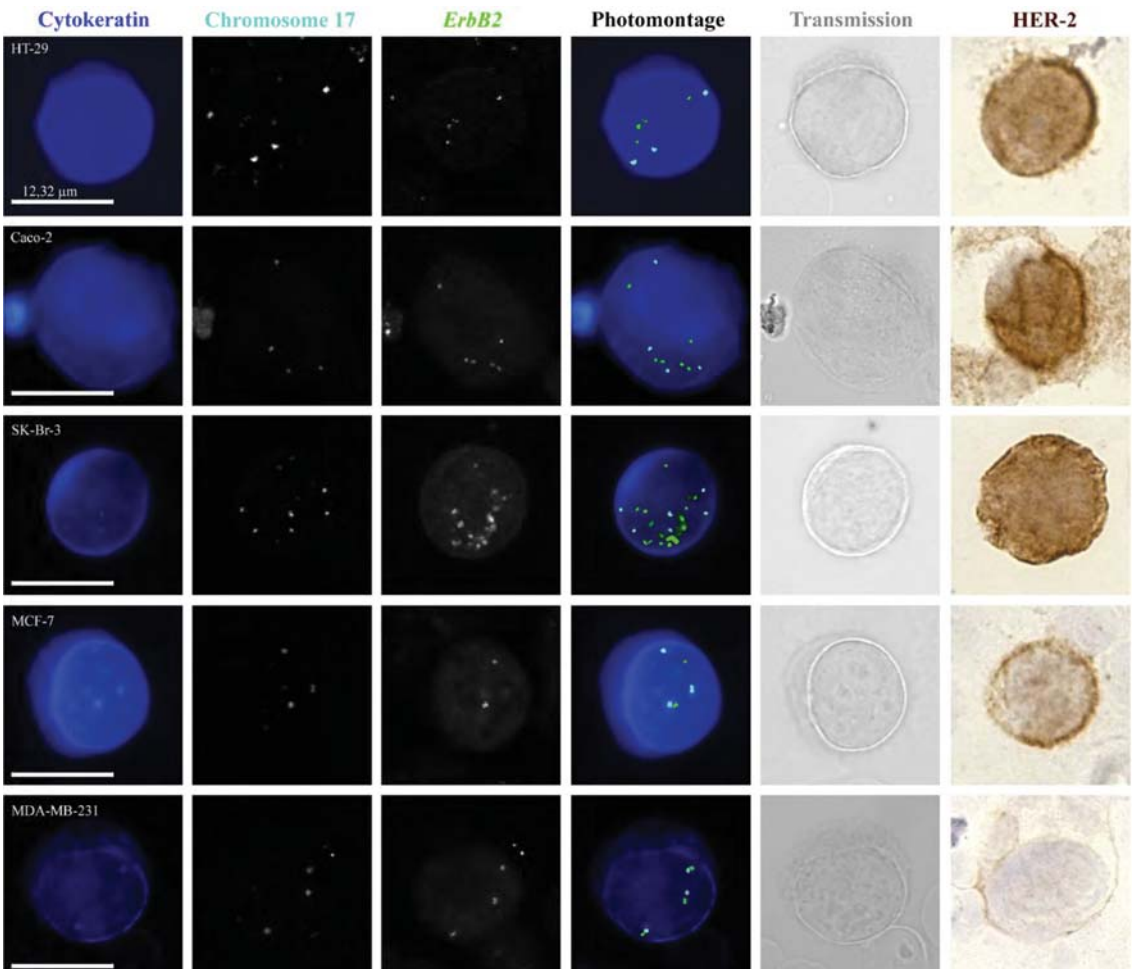

Fig. 1. Fluorescent images (x100 objective) of PB from healthy volunteers spiked with different tumor cell lines [HT-29, Caco-2, SK-Br-3, MCF-7 and MDA-MB-231] processed by immunomagnetic enrichment and FICTION techniques. Column "Cytokeratin" displays the CK labelling of tumor cells which are clearly 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
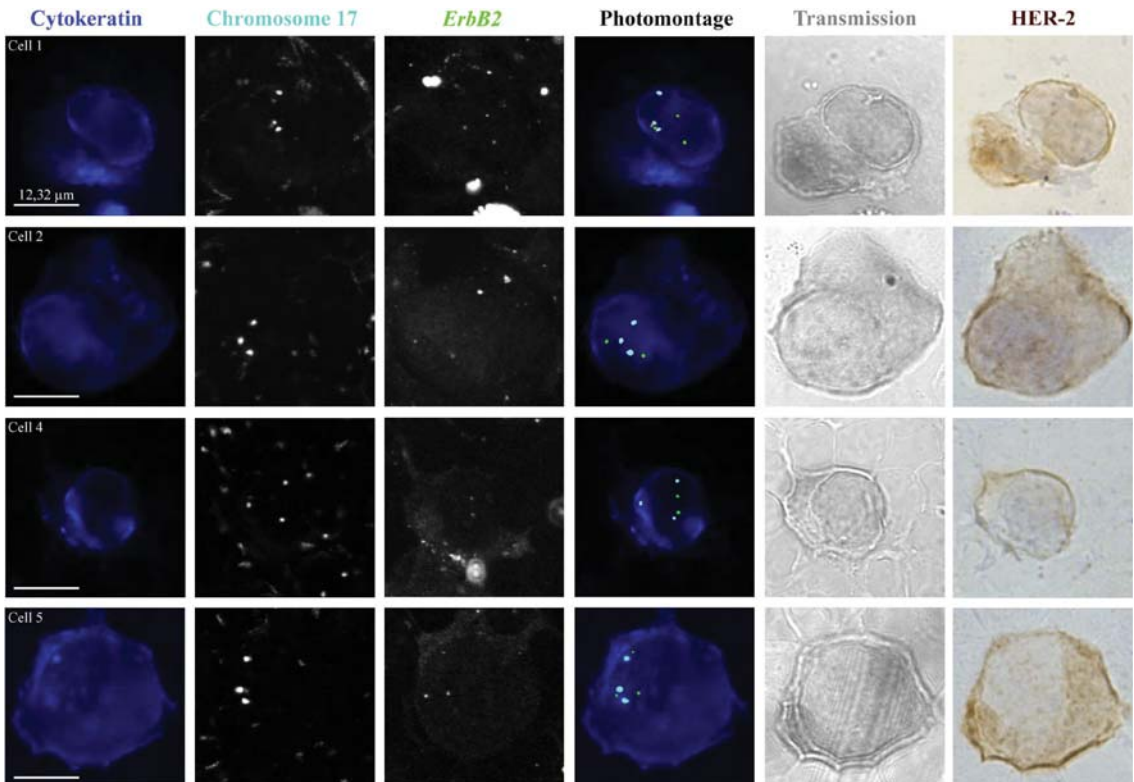

Fig. 2. Fluorescent images (x100 objective) of CK+/CTCs isolated from PB from IN 28 patient. Column "Cytokeratin" displays the CK labelling of these cells, which are clearly identified by their blue fluorescence. Samples were analyzed by fluorescence microscopy. Columns "Chromosome 17" and "ErbB2" show 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
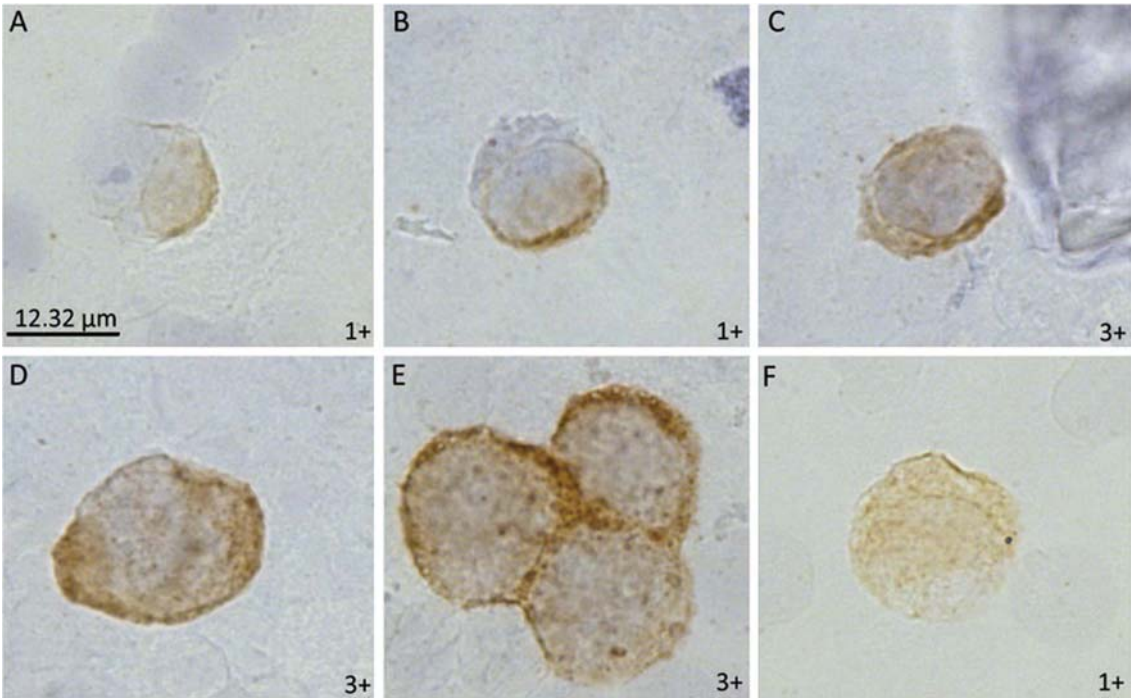
Fig. 3. HER2 expression on CK-/CTCs (x100 objective) from IN 28 patient. The score of HER2 protein expression is shown in the lower right corner of the images. Scale bar: 12.32 μm.
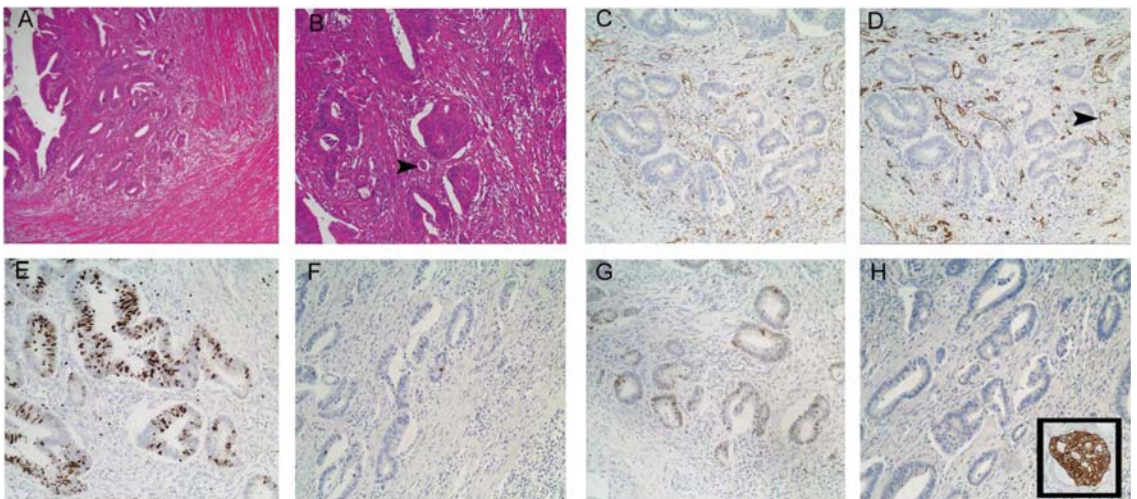

Fig. 4. Histopathological features of the tumor from IN 28 patient (stage II). **A.** Neoplastic glands with infiltrative pattern of growth affecting the muscular propria layer of the bowel. Haematoxylin-eosin. **B.** Detail from the neoplastic glands, atypical but well conformed, although fused glands 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.

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CURRICULUM VITAE

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1.- Titulación académica
Licenciatura en Biología
Universidad de Jaén. Facultad de Ciencias Experimentales
Finalización: Diciembre del 2008

2.- Otras titulaciones académicas
Máster en Olivar, Aceite de oliva y Salud
Fecha de finalización: Septiembre 2010

3.- Participación en Proyectos de Investigación

Título del proyecto: Estudio y aplicación de nuevas tecnologías para la mejora del proceso de extracción y obtención de aceite de oliva virgen con propiedades bioactivas y sensoriales singulares.
Entidad financiadora: INIA
Entidades participantes: Centro IFAPA Venta del Llano, Área de Inmunología, Universidad de Jaén
Duración, desde: 2008   hasta: 2011
Investigador responsable: Gabriel Beltrán Maza
Número de investigadores participantes: 10

Título del proyecto: Caracterización de propiedades biosaludables de los principales componentes minoritarios del aceite de oliva virgen (AOV) y, estudio de la inertización y control de atmósferas durante el batido de la pasta. Influencia en las características bioactivas y sensoriales del aceite. Potencial aplicación para el desarrollo de AOV con propiedades funcionales singulares.
Entidad financiadora: Conserjería de innovación, ciencia y empresa.
Entidades participantes: Centro IFAPA Venta del Llano, Área de Inmunología, Universidad de Jaén
Duración, desde: 2011   hasta: 2015
Investigador responsable: Gabriel Beltrán Maza
Número de investigadores participantes: 11

4.- Experiencia profesional hasta la fecha.

Beca de Inicio a la Investigación en la Universidad de Jaén (Plan de Apoyo)
Duración: Abril 2008 – Octubre 2008
Centro de Aplicación: Universidad de Jaén, Dpto. Ciencias de la Salud, Área de Inmunología

Beca de Colaboración del Ministerio de Educación, política Social y Deporte
Duración: Curso académico 2008 – 2009
Centro de Aplicación: Universidad de Jaén, Dpto. Ciencias de la Salud, Área de Inmunología

Fase de beca como Personal Investigador en Formación en la Universidad de Jaén
Duración: Marzo del 2010 – Febrero del 2012
Centro de Aplicación: Universidad de Jaén, Dpto. Ciencias de la Salud, Área de Inmunología

Fase de contrato como Personal Investigador en Formación en la Universidad de Jaén
Duración: Marzo 2012 – Febrero 2014
Centro de Aplicación: Universidad de Jaén, Dpto. Ciencias de la Salud, Área de Inmunología

5.- Participación en Seminarios, Congresos, Cursos y en Eventos de Difusión Científica relacionada con el Aceite de Oliva y Salud.

Denominación del evento: Congreso Internacional sobre Aceite de oliva y Salud "CIAS 2008"
Lugar de celebración y año: Jaén. España. 2008
Entidad/grupo organizador: CITOLIVA
Tipo de participación: Asistencia

Denominación del evento: XIV Simposium Cientifico-Técnico de EXPOLIVA 2009
Lugar de celebración y año: Jaén. España. 2009
Entidad/grupo organizador: Fundación para la Promoción y el Desarrollo del Olivar y del Aceite de Oliva
Tipo de participación: Comunicación Oral (Escualeno, componente minoritario del aceite de oliva virgen, y su acción en líneas tumorales y no tumorales de mama. Estudios "in vitro").

Denominación del evento: XIV Simposium Cientifico-Técnico de EXPOLIVA 2009
Lugar de celebración y año: Jaén. España. 2009
Entidad/grupo organizador: Fundación para la Promoción y el Desarrollo del Olivar y del Aceite de Oliva
Tipo de participación: Comunicación Oral (Protección del daño oxidativo al ADN del tirosol e hidroxitirosol, dos fenoles simples presentes en el aceite de oliva virgen extra, y su relación con el cáncer).

Denominación del evento: III Conferencia Internacional Encuentros en el Mediterráneo
Lugar de celebración y año: Instituto de la Alimentación Mediterránea. Málaga. España. 2009
Entidad/grupo organizador: Consejería de Agricultura y Pesca.
Tipo de participación: Asistencia a congreso

Denominación del evento: VI Congreso de la Sociedad Andaluza de Nutrición Clínica y Dietética
Lugar de celebración y año: Granada. 10, 11,12 Marzo 2010
Entidad/grupo organizador: Sociedad andaluza de nutrición clínica y dietética
Tipo de participación: Premio SAYCO al mejor póster "Actividad selectiva del escualeno, componente minoritario del aceite de oliva virgen extra entre líneas humanas tumorales y no tumorales de mama" presentado en el VI congreso de la SANCYD

Denominación del evento: VI Congreso de la Sociedad Andaluza de Nutrición Clínica y Dietética
Lugar de celebración y año: Granada. 10, 11,12 Marzo 2010
Entidad/grupo organizador: Sociedad andaluza de nutrición clínica y dietética
Tipo de participación: póster "Estudio de la capacidad antioxidante del hidroxitirosol, tirosol y escualeno, componentes minoriatrios del aceite de oliva virgen sobre células humanas de mama" presentado en el VI congreso de la SANCYD

Denominación del evento: Nutrition and Metabolism Nu.Me. 2nd International Mediterranean Meeting
Lugar de celebración y año: Granada. 16-19 Junio 2010
Entidad/grupo organizador: Sociedad andaluza de nutrición clínica y dietética
Tipo de participación: Póster "Antioxidant capacity of Squalene, the main hydrocarbon presents in Virgin Olive Oil, on a human breast epithelial cell line: MCF10A"

Denominación del evento: Nutrition and Metabolism Nu.Me. 2nd International Mediterranean Meeting
Lugar de celebración y año: Granada. 16-19 Junio 2010
Entidad/grupo organizador: Sociedad andaluza de nutrición clínica y dietética
Tipo de participación: Póster "Study in vitro of breast cancer metastasis prevention by some minor compounds presents in Virgin Olive Oil"

Denominación del evento: Influencia de los componentes minoritarios presentes en los aceites de oliva vírgenes en la prevención del cáncer de mama. Estudio del hidroxitirosol y tirosol
Lugar de celebración y año: Canena. Jaén. 2011
Entidad/grupo organizador: Castillo de Canena S.A.
Tipo de participación: 1º Premio

Denominación del evento: Acción del escualeno, componente minoritario del aceite de oliva virgen sobre células tumorales de mama
Lugar de celebración y año: Sevilla. 26-28 Octubre 2012
Entidad/grupo organizador: 31 Reunión nacional de la sección de ginecología oncológica y patología mamaria de la SEGO
Tipo de participación: Póster

Denominación del evento: XVII Convocatoria Premios a trabajos científicos e inéditos 2012 "Dr. Manuel Segovia Morón"
Lugar de celebración y año: Jaén, Octubre 2012
Tipo de participación: Premio al mejor trabajo
Entidad/grupo organizador: Ilustre Colegio Oficial de Médicos de la provincia de Jaén.

Denominación del evento: XIV Simposium Cientifico-Técnico de EXPOLIVA 2013
Lugar de celebración y año: Jaén. España. 2013
Entidad/grupo organizador: Fundación para la Promoción y el Desarrollo del Olivar y del Aceite de Oliva
Tipo de participación: Comunicación Oral (Disminución de la capacidad de invasión, implicada en el proceso metastásico de la línea tumoral mamaria MDA-MB-231 tras tratamiento con Tirosol).

Denominación del evento: XIV Simposium Cientifico-Técnico de EXPOLIVA 2013
Lugar de celebración y año: Jaén. España. 2013
Entidad/grupo organizador: Fundación para la Promoción y el Desarrollo del Olivar y del Aceite de Oliva
Tipo de participación: Comunicación Oral (Estudio in vitro de los efectos antiinflamatorios producidos por los principales triterpenos presentes en el aceite de oliva virgen).

Denominación del evento: Terceras Jornadas sobre investigación en biotecnología y biomedicina del máster universitario en biotecnología y biomedicina por la universidad de Jaén.
Lugar de celebración y año: Jaén, 19 Diciembre 2013
Entidad/grupo organizador: Máster Universitario en Biotecnología y Biomedicina por la Universidad de Jaén.

Denominación del evento: Conferenciante en Café con Ciencia 2013
Lugar de celebración y año: Jaén, 2013
Tipo de participación: Conferencia
Entidad/grupo organizador: Ciencia en Andalucía

Denominación del evento: Conferenciante en el Programa Campus científicos de verano 2013
Lugar de celebración y año: Jaén, Junio – Julio 2013
Tipo de participación: conferencia
Entidad/grupo organizador: Ministerio de Educación, Cultura y Deporte

Denominación del evento: Congreso científico de investigadores en formación en agroalimentación del CeiA3
Lugar de celebración y año: Jaén, Junio – Julio 2013
Tipo de participación: conferencia
Entidad/grupo organizador:

6. - <u>Publicaciones.</u>

Squalene protecs against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Warleta F, Campos M, Allouche Y, Sánchez-Quesada C, Ruiz-Mora J. Beltrán G, Gaforio JJ. *Food Chem Toxicol* 2010;
Tipo de producción: Artículo

Antioxidant, Antiproliferative, and Pro-apoptotic Capacies of Pentacyclic Triterpenes Found in the Skin if Olives on MCF-7 Human Breast Cancer Cells and Their Effects on DNA Damage. Allouche Y, Warleta F, Campos M, Sánchez-Quesada C, Uceda M, Beltrán G, Gaforio JJ. *J Agric Food Chem.* 2010.
Tipo de producción: Artículo

Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patiens. Campos M, Luque R, Jimenez J, Martinez R, Warleta F, Sanchez-Quesada C, Delgado Rodriguez M, Calvo A, Gaforio JJ. *Histology and histopathology.* 2013
Tipo de producción: Artículo

Hydroxytyrosol protects against oxidative DNA damage in human breast cells. Warleta F, Sánchez-Quesada C, Campos M, Allouche Y, Beltrán G, Gaforio JJ.2011
Tipo de producción: Artículo

Bioactive properties of the main triterpenes found in olives, virgin olive oil and leaves of *Olea Europaea.* Sánchez-Quesada C, López-Biedma A, Warleta F, Campos M, Beltrán G, Gaforio JJ.2013
Tipo de producción: Artículo

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. Gaforio JJ, Warleta F, Sánchez-Quesada C.
Tipo de producción: Libro

Olive oil, Mediterranean diet and health.Culture and knowledge inspired by olive oil.pp. 331 - 356.GEA Westfalia Separator Ibérica, SA. Gaforio JJ, Warleta F, Sánchez-Quesada C.
Tipo de producción: Capítulos de libros

Uso de la Electroforesis Unicelular Alcalina (Comet assay) para la identificación de componentes naturales de los alimentos con propiedades preventivas del daño oxidativo al ADN celular.Uso de la Electroforesis Unicelular Alcalina (Comet assay) para la identificación de componentes naturales de los alimentos con propiedades preventivas del daño oxidativo al ADN celular. Warleta F, Campos M, Sánchez-Quesada C, Gaforio JJ. 2012
Tipo de producción: Artículo

Aceite de oliva, dieta mediterránea y salud.Ad oleum habendum.pp. 295 - 322.GEA Westafalia Separator Ibérica. Gaforio JJ, Warleta F, Sánchez-Quesada C.
Tipo de producción: Capítulos de libros

7.- <u>Otros méritos.</u>

- Nivel Intermedio de inglés (B1) certificado por la E.O.I. (Escuela Oficial de Idiomas).
- Nivel Avanzado de inglés (B2) certificado por la E.O.I. (Escuela Oficial de Idiomas).
- Curso teórico sobre la utilización de microarrays (IVI Valencia)
- Curso Básico sobre el análisis de microarrays (IVI Valencia)