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TESIS DOCTORAL

CAPACIDAD ANTITUMORAL Y QUIMIOPREVENTIVA DEL PINORESINOL, PRINCIPAL LIGNANO PRESENTE EN LOS ACEITES DE OLIVA VÍRGENES, EVALUADO EN MODELOS CELULARES DE MAMA HUMANOS

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D. JOSÉ JUAN GAFORIO MARTÍNEZ, Profesor Titular del Departamento de Ciencias de la Salud de la Universidad de Jaén

CERTIFICA:

Que los trabajos llevados a cabo en la elaboración de la Tesis Doctoral titulada: "***Capacidad antitumoral y quimiopreventiva del pinoresinol, principal lignano presente en los aceites de oliva vírgenes, evaluado en modelos celulares de mama humanos***", presentada por Alicia López Biedma, han sido realizados bajo mi dirección, reuniendo las condiciones académicas necesarias para su presentación y defensa pública para optar al grado de Doctor.

En Jaén, a 10 de marzo de 2017

Fdo. Alicia López Biedma
Aspirante al grado de Doctor

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Abreviaturas

ADN: ácido desoxirribonucleico

AOV: aceite de oliva virgen

COX-2: ciclooxigenasa 2

CPH: complejo principal de histocompatibilidad

ECV: enfermedad cardiovascular

EGF: factor de crecimiento epidérmico (epidermal growth factor)

EGFR (o HER1): receptor 1 del factor de crecimiento epidérmico humano (human epidermal growth factor receptor-type 1)

EMT: transición epitelio-mesénquima (epitelial-mesenchymal transition)

ER: receptor de estrógenos (estrogen receptor)

EROs: especies reactivas del oxígeno

FGF: factor de crecimiento de fibroblastos (fibroblast growth factor)

GM-CSF: factor estimulador de colonias de granulocitos-monocitos (granulocyte-macrophage colony-stimulating factor)

H_2O_2: peróxido de hidrógeno

HER1: ver EGFR

HER2: receptor 2 del factor de crecimiento epidérmico humano (human epidermal growth factor receptor-type 2)

HIF-1: factor 1 inducible por hipoxia (hypoxia-inducible factor 1)

IFNγ: interferón gamma

ILs: interleucinas/citoquinas

LDL: lipoproteína de baja densidad (low-density lipoprotein)

LPS: lipopolisacáridos

NF-κβ: factor nuclear κβ (nuclear factor κβ)

MAPK: proteína quinasa activada por mitógeno (mitogen-activated protein kinase)

MEC: matriz extracelular

MIF: factor de inhibición de la migración de macrófagos (macrophage migration inhibitory factor)

MKP-1: proteína quinasa fosfatasa 1 activada por mitógeno (mitogen-activated protein kinase phosphatase 1)

MMP: metaloproteasas

MRC1: receptor de manosa, C tipo 1 (mannose receptor, C-type 1)

MUFAs: ácidos grasos monoinsaturados (monounsaturated fatty acids)

NO: óxido nítrico (nitric oxide)

PDGF: factor de crecimiento derivado de plaquetas (platelet-derived growth factor)

PGE_2: prostaglandina E2

PR: receptor de progesterona (progesterone receptor)

PUFAs: ácidos grasos poliinsaturados (polyunsaturated fatty acids)

RNS: especies reactivas del nitrógeno (reactive nitrogen species)

SFA: ácidos grasos saturados (saturated fatty acids)

SOD: superóxido dismutasa

TAFs: fibroblastos asociados a tumores (tumor-associated fibroblasts

TAMs: macrófagos asociados a tumores (tumor-associated macrophages)

TNF: factor de necrosis tumoral (tumor necrosis factor)

TSP-1: trombospondina 1 (thrombospondin 1)

VEGF: factor de crecimiento endotelial vascular (vascular endothelial growth factor)

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1. Introducción

1.1. Cáncer. Generalidades

La aparición de un tumor (tumorigénesis), o neoplasia, se debe al crecimiento anormal de tejido que no posee función fisiológica. Se trata de un proceso que comprende múltiples etapas en las que están involucradas alteraciones genéticas que conducen a la transformación progresiva de células normales en células con proliferación anormal. Si estas células no son capaces de infiltrarse e invadir otros tejidos, se les llama tumores benignos. Si por el contrario poseen capacidad metastásica se les denomina tumores malignos o, de forma general, cáncer. Así, podemos definir el cáncer como la enfermedad causada por células anormales y que pueden proliferar en una o varias partes del cuerpo[1,2].

A pesar de la enorme variedad de tipos y subtipos de cáncer, todas las células cancerosas comparten varias características[3], bien establecidas y que se detallan a continuación:

- *Mantenimiento de la señalización proliferativa*

  Los tejidos normales son capaces de controlar la producción y liberación al medio de señales, como factores de crecimiento que promueven el ciclo de división y el crecimiento celular, permitiendo un estado de homeostasis. Las células cancerosas,

[1] Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. 2016 Aug 11;18(1):84,016-0740-2.

[2] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70.

[3] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

por el contrario, mantienen una señalización proliferativa constante, y lo hacen generando sus propias señales de crecimiento (estimulación autocrina), provocando la secreción de dichas señales por parte de las células normales que forman el estroma tumoral, o interrumpiendo los mecanismos de feedback negativo responsables de atenuar la señal proliferativa.

- *Evasión de genes supresores del crecimiento*

  Las células tumorales, además, son capaces de sortear mecanismos que limitan el ciclo celular y la proliferación, lo que a menudo depende de genes supresores de tumores que están inactivados. La proteína RB (retinoblastoma) es un prototipo de supresor de tumores, ya que se encarga de integrar las diversas señales extra e intracelulares y, en respuesta, decide si una célula debe o no continuar con su ciclo de división y crecimiento. Otro prototipo es la proteína p53, que detiene el ciclo celular cuando el genoma está excesivamente dañado o los niveles de nucleótidos, glucosa, oxigenación y/o señales promotoras del crecimiento están por debajo de lo óptimo. Alternativamente, cuando el daño es irreparable, la p53 puede provocar apoptosis. A menudo, la inhibición del crecimiento por contacto célula-célula también está comprometida en las células tumorales, por lo que la proliferación continúa incluso a través de las membranas basales de tejidos y órganos adyacentes, perdiéndose la organización e integridad tisular.

- *Resistencia a la muerte celular*

  La apoptosis es un proceso esencial para el organismo en el que, ya sea por alteraciones en el ADN, insuficiencia de factores de supervivencia, hiperactividad de oncoproteínas u otras causas, la célula se desarma de forma progresiva y es finalmente fagocitada. La estrategia más común de las células tumorales para soslayar la apoptosis es la perdida de función de la p53, pero también lo

consiguen incrementando la expresión de reguladores antiapoptóticos o de supervivencia (como Bcl-2) o disminuyendo los proapoptóticos (como Bax), entre otros mecanismos.

- *Inmortalidad replicativa*

  Otra característica propia de células sanas y que no presentan las células tumorales es el acortamiento de los telómeros. Estos son repeticiones de nucleótidos que protegen las terminaciones de los cromosomas y que se van acortando en cada ciclo celular, lo que conduce a la senescencia, un estado irreversible en el que la célula es viable pero no prolifera.

- *Angiogénesis sostenida*

  Al igual que el tejido sano, los tumores requieren nutrientes y oxígeno, así como eliminar desechos metabólicos y CO_2. La diferencia reside en que la formación de vasos sanguíneos a partir de preexistentes (angiogénesis) se encuentra activada de forma crónica en el caso de los tumores. Sobre este hecho profundizaremos posteriormente.

- *Activación de la invasión y metástasis*

  De forma adicional, algunas células cancerosas pueden desarrollar diversas alteraciones, como la pérdida de la molécula de adhesión celular E-cadherina, para producir la invasión de tejidos adyacentes y culminar con la metástasis, procesos a los cuales haremos referencia más adelante.

- *Reprogramación del metabolismo energético*

  Para obtener energía, las células normales llevan a cabo la oxidación de la glucosa hasta piruvato. En condiciones aeróbicas (glucólisis aerobia), este pasa a la mitocondria, donde se produce la fosforilación oxidativa obteniéndose CO_2 y H_2O. En

condiciones anaeróbicas (glucólisis anaeróbica) permanece en el citosol de la célula y es reducido hasta lactato. A pesar de que el balance energético es mucho mayor en el primer caso (hasta 36 moléculas de ATP frente a 2 moléculas de ATP), las células cancerosas realizan la conversión de glucosa en lactato, incluso en presencia de suficiente oxígeno (efecto Warburg). Esta ineficiencia energética no parece ser un obstáculo, dado que la angiogénesis aberrante que tienen asociada aporta las fuentes de energía necesarias y, además, la producción de lactato estimula la angiogénesis, la supervivencia celular y la proliferación a través de diferentes mecanismos[4].

- *Evasión de la inmunidad*

  La teoría de la "Inmunoedición del cáncer" pone de manifiesto tres posibles desenlaces para la interacción entre el sistema inmune y el tumor[5]:

  - Eliminación: la inmunidad (innata y adaptativa) destruye el tumor en desarrollo.
  - Equilibrio: la inmunidad adaptativa no elimina el tumor, pero frena su expansión hasta un estado de latencia.
  - Escape: la expansión deja de ser bloqueada y la enfermedad se manifiesta clínicamente. Este último caso puede producirse gracias a diversos mecanismos, como la pérdida o disfunción de antígenos para el reconocimiento por parte de la inmunidad, el aumento de la resistencia a

[4] Tran Q, Lee H, Park J, Kim SH, Park J. Targeting Cancer Metabolism - Revisiting the Warburg Effects. Toxicol Res. 2016 Jul;32(3):177-93.

[5] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011 Mar 25;331(6024):1565-70.

sus efectos citotóxicos con el incremento, por ejemplo, de mecanismos antiapoptóticos y factores de transcripción prooncogénicos, o la inducción de un estado inmunosupresivo por la producción de citoquinas.

En relación a esto último y, una vez desgranados los sellos distintivos de las células tumorales, hay que tener presente que el cáncer no se limita a las células malignas propiamente dichas, sino que su complejidad alcanza numerosos procesos patofisiológicos que son cruciales para el inicio y la progresión del tumor, como el reclutamiento de células normales al estroma tumoral, la modulación de células mieloides, en particular macrófagos, o la creación de zonas hipóxicas en el llamado "microambiente tumoral", como se explicará posteriormente[6,7].

1.1.1. Carcinogénesis: factores para el desarrollo de tumores

En la mayor parte de los cánceres intervienen varios factores etiopatogénicos. Muchos agentes químicos y radiaciones modifican el ADN y actúan como agentes mutagénicos, cuyos mecanismos de acción confluyen en un pequeño número de procesos que alteran regiones del ADN y que provocan que los oncogenes estén estimulados, los genes supresores, inhibidos funcionalmente o delecionados, y los genes promotores de oncogenes, mutados. Conforme la exposición es más prolongada, más frecuente o más intensa, la probabilidad de que se produzca cáncer es mayor, aunque

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[6] Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA. Hypoxia and tumor-associated macrophages: A deadly alliance in support of tumor progression. Oncoimmunology. 2014 Jan 1;3(1):e27561.

[7] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

pequeñas exposiciones o incluso una exposición única pueden llegar a producir una neoplasia. Las mutaciones aumentan en los casos en que existe predisposición genética, o procesos bioquímicos o genéticos relacionados con la disminución de enzimas inhibidoras de productos cancerígenos activos, el aumento de enzimas activadoras de genes cancerígenos o fallos en los procesos de reparación de las alteraciones del ADN, así como factores de facilitación (factores hormonales, deficiencias inmunológicas, actividad de las enzimas de reparación del ADN, etc.)[8].

El 85% de las enfermedades cancerosas tienen como causa factores exógenos, entre los que podemos encontrar:

- Agentes físicos:
  Entre las radiaciones ionizantes (aquellas que causan la separación de electrones de átomos y moléculas) se encuentran los neutrones, los rayos X y los rayos γ, y menos frecuentemente las radiaciones β y α de los radioisótopos. Las fuentes de estas radiaciones van desde los rayos cósmicos, los componentes de la corteza terrestre (uranio) y los materiales de construcción (radón) hasta los accidentes de centrales nucleares o la exposición tanto profesional (minería, pinturas, etc.), como médica y diagnóstica. Las únicas radiaciones no ionizantes importantes capaces de producir cáncer son los rayos UV, siendo su principal fuente de exposición la luz solar. Otros factores físicos son la irritación crónica y ciertos cuerpos extraños como talco o amianto.

- Agentes químicos:
  La lista aquí es muy extensa, pero probablemente esté encabezada por el tabaco, que causa el 30% de los fallecimientos por cáncer. Entre los agentes químicos

[8] Valladares Sánchez Y. Historia natural del cáncer. Causas, procesos y manifestaciones. Madrid: Rescate; 2007.

cancerígenos se incluyen agentes alquilantes, hidrocarburos aromáticos policíclicos, aminas aromáticas, colorantes azoicos, nitrosocompuestos, micotoxinas, cloruro de vinilo y dioxina.

- Agentes biológicos:
  Los virus oncogénicos son responsables del 15% de los tumores humanos, y entre ellos cabe destacar los virus de la hepatitis B y C, el papilomavirus humano y el virus de Epstein-Barr causantes, respectivamente, de carcinoma hepatocelular, cáncer de cuello uterino y linfomas de Burkitt y Hodgkin.
  Algunos parásitos también son capaces de producir cáncer, con uno de los casos más evidentes en la asociación entre la esquistosomiasis y el cáncer de vejiga urinaria.

- Factores alimenticios:
  Consumo de alcohol y otros productos, que además llevan al sobrepeso y la obesidad, y a los que haremos especial mención posteriormente.

Respecto a los factores endógenos, se incluyen:

- Edad: conforme aumenta, también lo hace la incidencia de cáncer, debido al acúmulo de mutaciones.

- Factores genéticos y de herencia: muchas veces ligados a la raza, como ocurre con la baja incidencia de cáncer de mama en mujeres japonesas, a desequilibrios hormonales, a inmunodeficiencias hereditarias o a mutaciones y alteraciones genéticas que predisponen o están directamente relacionadas con el cáncer.

- Factores hormonales: de especial importancia, por ejemplo, en cáncer de mama, ya que los estrógenos ocasionan una

hiperplasia crónica que puede ser la antesala de muchos casos en este tipo de cáncer.

- ❖ Estrés oxidativo e inflamación crónica: a menudo estos dos procesos, que pueden aparecer por multitud de causas, están interconectados y predisponen al tejido a un proceso tumorigénico, como se explica a continuación.

1.1.1.1. Estrés oxidativo y cáncer

La producción de radicales libres, tanto las especies reactivas del oxígeno (EROs) como las del nitrógeno (RNS), es esencial para la defensa frente a microorganismos y para la homeostasis celular, actuando estos radicales como mensajeros secundarios necesarios en procesos fisiológicos normales. Sin embargo, cuando el balance entre estas especies reactivas y los mecanismos antioxidantes que las eliminan se inclina a favor de las primeras, el estrés oxidativo/nitrosativo causado puede desembocar en la disfunción y muerte de la célula y, a la larga, en daño tisular y fallo orgánico. De hecho, numerosas patologías como hipertensión, aterosclerosis, Alzheimer, Parkinson, procesos de envejecimiento o cáncer, están asociadas a la presencia de radicales libres. Los daños que provocan a las células incluyen lipoperoxidación de ácidos grasos poliinsaturados, oxidación proteica, daño al ADN, oxidación del ARN, despolarización mitocondrial o apoptosis[9].

En relación a la carcinogénesis, probablemente la diana más importante es el ADN, que puede sufrir la oxidación de las bases nitrogenadas, la rotura de hebras, el intercambio de cromátidas

[9] Oter S, Jin S, Cucullo L, Dorman HJ. Oxidants and antioxidants: friends or foes? Oxid Antioxid Med Sci. 2012;1(1):1-4.

hermanas o la formación de micronúcleos[10]. Pero el estrés oxidativo también puede contribuir al inicio del cáncer a través de rutas de señalización protumorigénicas, como ocurre con la oxidación del supresor de tumores PTEN o la inactivación de la proteína quinasa activada por mitógeno (MAPK)[11].

Las EROs se generan tanto por fuentes exógenas (contaminantes, tabaco, radiaciones, etc., vistas anteriormente) como por fuentes celulares endógenas, e incluyen las especies radicales anión superóxido ($O_2^{\cdot-}$), peróxido de hidrógeno (H_2O_2) y radical hidroxilo ($OH^{\cdot}$). A nivel celular, el superóxido se genera principalmente mediante la reducción del oxígeno molecular en la mitocondria a través de la cadena de electrones o en la matriz extracelular (MEC) a través de la familia de las NADPH oxidasas (NOXs). Este, a su vez, puede convertirse en H_2O_2 en la matriz mitocondrial (gracias a la superóxido dismutasa 2, SOD2), en el citosol (SOD1) o en la matriz extracelular (SOD3). Se trata de la forma más estable de EROs y puede afectar a la señalización celular interactuando con los residuos de cisteína de moléculas diana. Los niveles normales de H_2O_2 (rango nanomolar bajo) permiten mantener la homeostasis, que se pierde cuando esta concentración óptima disminuye. Por el contrario, si los niveles aumentan, puede aparecer el daño oxidativo y las patologías que ya hemos mencionado, o la muerte celular en el caso de niveles muy altos. Si, además, el H_2O_2 reacciona con iones de hierro o cobre (Fe^{2+}, Cu^{2+}), se produce el radical $OH^{\cdot}$, extremadamente reactivo y dañino para lípidos, proteínas y ADN. En el lado opuesto, la enzima catalasa

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[10] Lopaczynski W, Zeisel SH. Antioxidants, programmed cell death, and cancer. Nutr Res. 2001 0;21(1–2):295-307.

[11] Nogueira V, Hay N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin Cancer Res. 2013 Aug 15;19(16):4309-14.

o los sistemas glutatión (GSH) y tiorredoxina (TRX) pueden convertir el H_2O_2 en agua[12].

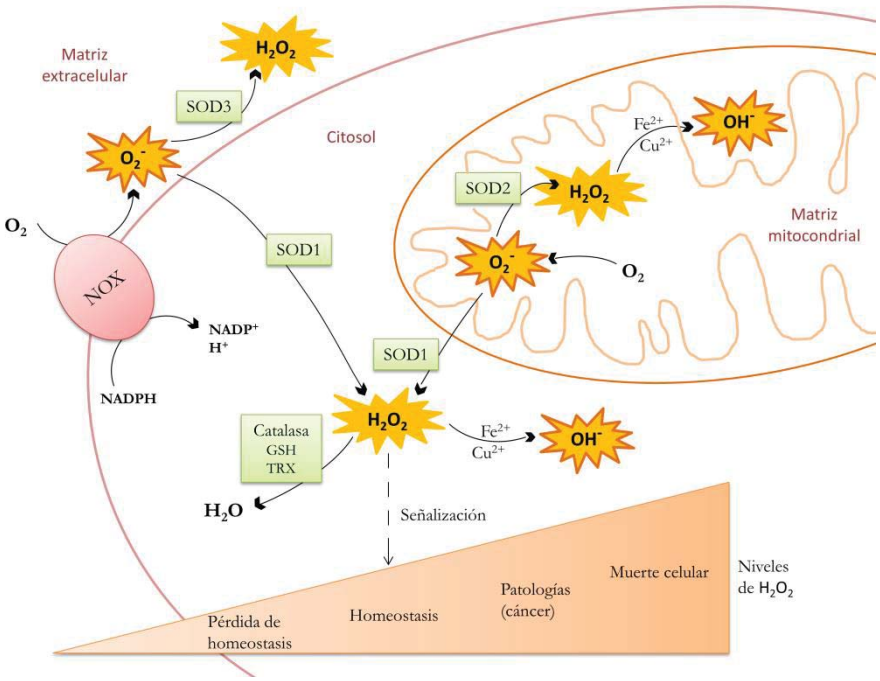

Figura 1. Formación y localización de EROs (modificada de Glasauer y Chandel 2014)[13]

Por tanto, los efectos perjudiciales de las EROs están moderados por enzimas endógenos y por sistemas reguladores, pero también por

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[12] Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. Biochem Pharmacol. 2014 Nov 1;92(1):90-101.

antioxidantes que tienen que ser incorporados a través de la dieta, como ácido ascórbico, α-tocoferol, β-caroteno, isoflavonas, etc.[14,15].

Podemos definir a los antioxidantes como compuestos capaces de donar electrones y neutralizar a los radicales libres o compuestos oxidantes, los cuales son átomos con uno o más electrones desapareados que presentan gran inestabilidad y reactividad en el intento de obtener dichos electrones de otros compuestos. El resultado es una molécula menos agresiva que el agente oxidante inicial[16]. Por este motivo, resulta fundamental el aporte de antioxidantes mediante la ingesta de frutas, verduras, legumbres y frutos secos como parte de la dieta en la prevención del cáncer.

Existe, sin embargo, gran controversia acerca del uso de suplementos nutricionales antioxidantes en pacientes de cáncer. Las células cancerosas, en comparación con las normales, se caracterizan por presentar niveles elevados de EROs con objeto de activar vías de señalización que promuevan la proliferación, la supervivencia y la adaptación metabólica al microambiente tumoral[17]. Aprovechando este hecho, muchos agentes quimioterapéuticos se basan en la producción de EROs hasta niveles que las células cancerosas son incapaces de

[14] Nogueira V, Hay N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin Cancer Res. 2013 Aug 15;19(16):4309-14.

[15] Lopaczynski W, Zeisel SH. Antioxidants, programmed cell death, and cancer. Nutr Res. 2001 0;21(1–2):295-307.

[16] Saeidnia S, Abdollahi M. Antioxidants: friends or foe in prevention or treatment of cancer: the debate of the century. Toxicol Appl Pharmacol. 2013 Aug 15;271(1):49-63.

[17] Nogueira V, Hay N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin Cancer Res. 2013 Aug 15;19(16):4309-14.

tolerar, pero que no llegan a ser tóxicos para las células normales gracias a la acción de las enzimas antioxidantes (ineficientes en dichas células cancerosas), o bien en la disminución o inactivación de los mecanismos antioxidantes que las células tumorales pueden desarrollar precisamente para adaptarse a estas condiciones[18]. Puesto que este exceso es capaz de inducir apoptosis y detener el ciclo celular, su inhibición por parte de los antioxidantes podría interferir en la eliminación de las células cancerosas[19]. Así, los antioxidantes, que en individuos sanos juegan un papel protector frente al daño oxidativo, podrían promover el cáncer en individuos con estado precanceroso y pacientes con cáncer, mediante la inhibición de la apoptosis. Dicho de otro modo, las concentraciones elevadas de EROs pueden considerarse oncogénicas, pero también pueden producir la muerte de células tumorales o suprimir su crecimiento y progresión, por lo que son frecuentes las referencias a ellas como "armas de doble filo"[18]. Además del estado de progresión del cáncer, parece ser que el papel preventivo de los antioxidantes también depende de la diana del propio antioxidante y de las dosis a las que se encuentre.

1.1.1.2. Inflamación crónica y cáncer

Existen diferentes estímulos que pueden ocasionar daño en un individuo, incluyendo virus, bacterias, factores alimenticios, contaminantes medioambientales e incluso estrés. Parte de la respuesta del organismo frente a estas agresiones, para contrarrestar los daños producidos, es lo que conocemos como inflamación, la cual Cornelius Celsus, ya en el año 40 d. C., identificó como la manifestación de

[18] Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. Biochem Pharmacol. 2014 Nov 1;92(1):90-101.

[19] Lopaczynski W, Zeisel SH. Antioxidants, programmed cell death, and cancer. Nutr Res. 2001 0;21(1–2):295-307.

cuatro signos: "rubor, calor, dolor y tumefacción"[20]. Un quinto signo fue añadido por Rudolf Virchow (1821-1902), haciendo referencia a la "pérdida o disminución de la función" en los tejidos[21].

En realidad, estos signos son la manifestación macroscópica del proceso, que incluye señales para la instrucción celular, la destrucción de agentes infecciosos y de células infectadas o dañadas, la licuefacción del tejido adyacente y la reparación de la zona afectada[22].

La inflamación aguda, que se manifiesta en un periodo corto de tiempo, tiene por tanto una función terapéutica. Sin embargo, cuando esta respuesta pasa a ser crónica o demasiado larga, puede ocasionar perjuicios y conducir a la enfermedad. Así, por ejemplo, la diabetes, la artritis, el Alzheimer y enfermedades cardiovasculares o autoinmunes están mediadas por la inflamación crónica. Al igual, esta es un factor de riesgo para muchos tipos de cáncer, y se le relaciona con varios procesos en la formación de tumores, tales como transformación celular, supervivencia, proliferación, invasión, angiogénesis y metástasis[23]. Algunas de las neoplasias asociadas a condiciones de inflamación crónica son, por ejemplo, el melanoma en el caso de la inflamación de la piel, el carcinoma colorectal en la enfermedad de

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[20] Nathan C. Points of control in inflammation. Nature. 2002 Dec 19-26;420(6917):846-52.

[21] Heidland A, Klassen A, Rutkowski P, Bahner U. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? J Nephrol. 2006 May-Jun;19 Suppl 10:S102-9.

[22] Nathan C. Points of control in inflammation. Nature. 2002 Dec 19-26;420(6917):846-52.

[23] Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol. 2006 Nov 30;72(11):1605-21.

Crohn y en la colitis ulcerosa, el hepatocarcinoma en la hepatitis o el carcinoma pulmonar en la bronquitis crónica[24].

La inmunidad innata comprende diferentes tipos de células, como los linfocitos citolíticos naturales (NK), encargados de lisar células infectadas y activar a los macrófagos, y los fagocitos. A este segundo grupo corresponden los neutrófilos, que fagocitan y destruyen agentes infecciosos en las primeras fases de las respuestas inflamatorias, los macrófagos, que lo hacen en etapas posteriores de la infección y activan a otras células del sistema inmune, y las células dendríticas, que fagocitan agentes infecciosos y activan a los linfocitos T.

De entre todos ellos, los macrófagos juegan un papel fundamental en cáncer de mama, puesto que llegan a formar hasta el 80% de la masa tumoral[25]. Estos macrófagos se originan en los tejidos a partir de la diferenciación de monocitos precursores presentes en el torrente sanguíneo, que a su vez proceden de células progenitoras (monoblastos) de la médula ósea. Además de la extravasación de monocitos circulantes, un pequeño porcentaje de macrófagos (5%) deriva de la división local de fagocitos mononucleares en los tejidos[26].

Una vez diferenciados a macrófagos, y dependiendo de las señales que reciban en el microambiente donde se encuentran, estos se activan en un amplio rango de estados, que a menudo se simplifican con los términos M1 (o macrófagos activados por vía clásica) y M2 (o

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[24] Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002 Dec 19-26;420(6917):860-7.

[25] Allavena P, Sica A, Garlanda C, Mantovani A. The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev. 2008 Apr;222:155-61.

[26] Abbas KA, Lichtman HA, Pillai S. Inmunología celular y molecular. Barcelona, España. Elsevier. 2008;6.ª edición.

macrófagos alternativamente activados), basándose, respectivamente, en la alta o baja expresión de moléculas del complejo principal de histocompatibilidad (CPH) de clase II[27,28].

La polarización a M1 aparece como respuesta a productos bacterianos como los lipopolisacáridos (LPS), a la secreción de IFNγ (Interferón gamma) por parte de los linfocitos Th-1 y NK, a la expresión de CD40L (ligando de la proteína de membrana CD40) en los Th-1 y a la presencia en el medio de otros factores como TNF (factor de necrosis tumoral) y GM-CSF (factor estimulador de colonias de granulocitos-monocitos).

Este fenotipo M1 está orientado a fagocitar y eliminar células diana y desechos celulares a través de la producción de enzimas proteolíticas, EROs e intermediarios reactivos del nitrógeno, en especial el óxido nítrico (NO) que, como ya hemos mencionado, contribuyen al proceso de apoptosis. Además de su claro papel en la eliminación de agentes externos, son capaces de fagocitar $2x10^{11}$ eritrocitos/día, lo que supone alrededor de 3 kg de hierro y hemoglobina/año que son reutilizados en un proceso metabólico vital para el hospedador, así como de retirar restos procedentes del remodelado tisular y de células que han llevado a cabo apoptosis o necrosis[29]. Además, los M1 se encargan de atraer y activar otros monocitos y células del sistema

[27] Heusinkveld M, Van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med. 2011 Dec 16;9:216,5876-9-216.

[28] Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA. Hypoxia and tumor-associated macrophages: A deadly alliance in support of tumor progression. Oncoimmunology. 2014 Jan 1;3(1):e27561.

[29] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008 Dec;8(12):958-69.

inmune adaptativo mediante la liberación de citoquinas pro-inflamatorias como IL-1β, IL-6, IL-12, IL-15, IL-23 y TNF[30,31].

Por el contrario, la polarización a M2 se da cuando las señales recibidas son las citoquinas IL-4, IL-10 o IL-13, glucocorticoides u hormonas secosteroides (Vitamina D_3). Este fenotipo se focaliza en la reestructuración tisular y la resolución de la herida, agresión o infección, por lo que expresa abundantes receptores "scavenger" (MSR1/CD204), receptores de manosa (MRC1/CD206), metaloproteasas (MMP) encargadas de degradar la matriz extracelular, factores de crecimiento de fibroblastos y angiogénicos como VEGF y citoquinas antiinflamatorias como IL-10[32,33].

[30] Abbas KA, Lichtman HA, Pillai S. Inmunología celular y molecular. Barcelona, España. Elsevier. 2008;6.ª edición.

[31] Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol. 2006 Nov 15;177(10):7303-11.

[32] Abbas KA, Lichtman HA, Pillai S. Inmunología celular y molecular. Barcelona, España. Elsevier. 2008;6.ª edición.

[33] Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol. 2006 Nov 15;177(10):7303-11.

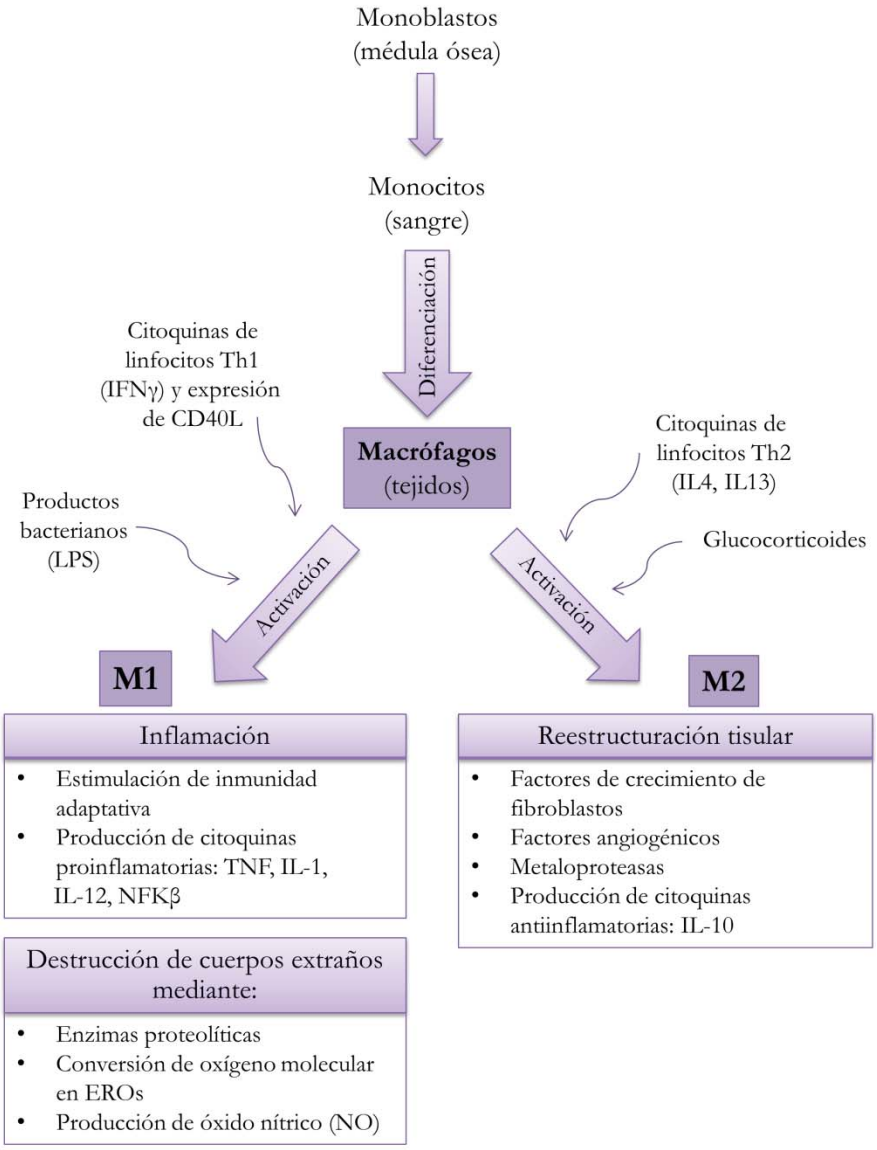

Figura 2. Esquema de la diferenciación y activación de las poblaciones M1 y M2 de los macrófagos y sus respectivas funciones.

Como ya se ha mencionado, la inflamación, y en primer plano la actividad proinflamatoria de los macrófagos activados a M1, pueden predisponer al tejido circundante a una transformación neoplásica, debido fundamentalmente a que los radicales libres que producen pueden causar daño en el ADN[34], aunque una vez establecido el tumor, estudios *in vitro* han descrito la habilidad de los M1 para eliminar células tumorales[35].

Por el contrario, en ese ambiente tumoral, el cual requiere nutrientes, oxígeno y la posibilidad de desechar productos metabólicos y dióxido de carbono, los macrófagos M2 son una herramienta perfecta para la progresión del tumor, puesto que producen factores que impiden la función de los linfocitos Th-1 y que promueven la vascularización y la invasión y metástasis de las células cancerosas[36]. De hecho, los llamados macrófagos asociados a tumores (TAMs), es decir, macrófagos que están infiltrados en el tumor, pertenecen en su mayoría a este último fenotipo y su presencia en cáncer de mama, entre otros tumores, se ha relacionado con un mal pronóstico y con la progresión de la enfermedad[37,38].

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[34] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008 Dec;8(12):958-69.

[35] Braster R, O'Toole T, van Egmond M. Myeloid cells as effector cells for monoclonal antibody therapy of cancer. Methods. 2014 Jan 1;65(1):28-37.

[36] Heusinkveld M, Van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med. 2011 Dec 16;9:216,5876-9-216.

[37] Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res. 2006 Jan 15;66(2):605-12.

No obstante, el microambiente tumoral es un compendio de distintas subpoblaciones de macrófagos que cubren un espectro más amplio que el presentado en la clasificación simplificada entre M1 y M2, existiendo una gran heterogeneidad de macrófagos que además pueden presentar plasticidad, es decir, pueden pasar de un estado de activación a otro en función de las señales que reciban. Así, la evolución del tumor dependerá en gran medida de la comunicación entre las células tumorales y los macrófagos, además de otros tipos celulares presentes como linfocitos, en un pulso por adoptar características proinflamatorias o antiinflamatorias.

1.1.2. Evolución y progresión del cáncer

1.1.2.1. El microambiente tumoral

Como acabamos de señalar, el microambiente tumoral lo conforman varios tipos celulares que interaccionan con las células tumorales, así como una red de arterias, venas y capilares, y la matriz extracelular (MEC). Ya hemos profundizado en el papel de los macrófagos en este ambiente, pero también aparecen otras células de la inmunidad innata (neutrófilos, células dendríticas, células NK), de la inmunidad adaptativa (linfocitos B y T) y células estromáticas (fibroblastos, células endoteliales, pericitos, adipocitos y células madre mesenquimales) que, de hecho, pueden llegar a formar hasta el 80% de la masa tumoral[39,40].

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[38] Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int J Dev Biol. 2011;55(7-9):861-7.

[39] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

Todos estos componentes mantienen una "conversación" basada en factores de crecimiento, quimiocinas, citoquinas, etc., y enfocada siempre al avance del tumor. La interacción llega hasta tal punto que puede ocurrir transdiferenciación de células del estroma a otro tipo de células estromales, pero también de células cancerosas a estromales, las cuales serán capaces de aportar ciertos requerimientos para la progresión tumoral[41]. Pese a esto, parece ser que el punto más vulnerable de este microambiente está en ciertas células inmunitarias que sí son capaces de destruir el tumor (macrófagos M1, subploblaciones de células NK y de linfocitos B y T) y que adquieren enorme importancia en la prognosis y en el diseño de posibles terapias enfocadas a dirigir a estas células hacia la eliminación tumoral. El microambiente tumoral se caracteriza, además, por la acidificación que resulta de la producción de enormes cantidades de lactato, el cual contribuye a la angiogénesis, la inmunosupresión y la metástasis[42].

1.1.2.2 Vascularización tumoral

La angiogénesis implica proliferación, migración y morfogénesis de células endoteliales. Se trata de un proceso fundamental durante el desarrollo y en situaciones fisiológicas como la cicatrización de heridas o el ciclo menstrual, pero es también esencial para la progresión

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[40] Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. 2016 Aug 11;18(1):84,016-0740-2.

[41] Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. 2016 Aug 11;18(1):84,016-0740-2.

[42] Romero-Garcia S, Moreno-Altamirano MM, Prado-Garcia H, Sanchez-Garcia FJ. Lactate Contribution to the Tumor Microenvironment: Mechanisms, Effects on Immune Cells and Therapeutic Relevance. Front Immunol. 2016 Feb 16;7:52.

tumoral, tanto por el aporte de oxígeno y nutrientes como por la oportunidad de diseminación que supone para las células metastásicas[43]. De igual modo, la formación de nuevos vasos linfáticos, conocida como linfangiogénesis, es aprovechada por las células tumorales para su propagación, pudiendo alojarse en los ganglios linfáticos adyacentes y, posteriormente, producir metástasis en ganglios distantes y/o en órganos a través del conducto torácico o el sistema sanguíneo[44].

En la angiogénesis y la linfangiogénesis tumoral existen señales concretas que favorecen ambos procesos, producidas tanto por células tumorales como por TAMs o fibroblastos asociados al tumor (TAFs), y cuyo mayor exponente es el VEGF (factor de crecimiento endotelial vascular). Otros inductores son el factor de crecimiento derivado de plaquetas (PDGF), el factor de crecimiento de fibroblastos (FGF), IL-8 o angiopoyetinas. Por otra parte, hay una carencia de factores de maduración e inhibidores de la angiogénesis como endostatina, angiostatina o trombospondina 1 (TSP-1)[45].

El resultado de estas señales es la formación desorganizada, casi caótica, de nuevos vasos sanguíneos y linfáticos anormales, tortuosos, que presentan finales ciegos y revestimientos endoteliales y membranas basales incompletas. En ciertas áreas, por tanto, hay un gran volumen de flujo y presión intersticial, pero en otras, el flujo

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[43] Bielenberg DR, Zetter BR. The Contribution of Angiogenesis to the Process of Metastasis. Cancer J. 2015 Jul-Aug;21(4):267-73.

[44] Pastushenko I, Conejero C, Carapeto FJ. Lymphangiogenesis: implications for diagnosis, treatment, and prognosis in patients with melanoma. Actas Dermosifiliogr. 2015 Jan-Feb;106(1):7-16.

[45] Wang Z, Dabrosin C, Yin X, Fuster MM, Arreola A, Rathmell WK, et al. Broad targeting of angiogenesis for cancer prevention and therapy. Semin Cancer Biol. 2015 Dec;35 Suppl:S224-43.

sanguíneo es lento e irregular. En consecuencia, aparecen regiones hipóxicas (0.1-1 mm Hg) e incluso anóxicas (falta total de oxígeno), en contraposición a los valores de tensión de oxígeno que suelen presentar los tejidos normales (entre 30 y 70 mm Hg). La falta de nutrientes y oxígeno en estas zonas induce factores de transcripción como el factor 1 inducible por hipoxia (HIF-1), que regula genes relacionados con la glucólisis e inhibe la respiración mitocondrial, por lo que las células tumorales pasan a producir energía mediante glucólisis anaerobia. A pesar de que, en un principio, las células tumorales no proliferan en estas regiones de hipoxia, la presencia de las mismas está asociada a un mal pronóstico, tanto por la resistencia que ofrecen a las terapias antitumorales (los radicales libres derivados del oxígeno potencian el daño a proteínas y ADN producido por la radiación ionizante) como por la selección sobre las propias células tumorales (solo sobreviven aquellas con fenotipo agresivo, capaces de superar esas condiciones, de repoblar el tumor y de producir metástasis)[46]. Además, se ha demostrado que aunque la hipoxia no es la principal inductora de la activación de los TAMs, sí que potencia la actividad biológica y, en concreto, las funciones angiogénicas de los M2, que suelen localizarse en estas regiones hipóxicas[47].

[46] Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood. 2004 Oct 15;104(8):2224-34.

[47] Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA. Hypoxia and tumor-associated macrophages: A deadly alliance in support of tumor progression. Oncoimmunology. 2014 Jan 1;3(1):e27561.

1.1.2.3 Metástasis

Cada fase de la progresión tumoral conlleva condiciones adversas que eliminan a la mayoría de las células cancerosas, pero algunos tumores malignos albergan células con los rasgos necesarios para vencer estos obstáculos. Si dichas células tienen capacidad propagadora (actuando como célula madre de cáncer) y la presencia de vascularización lo permite, se producirá la metástasis. Su importancia clínica, como fase final de la enfermedad, radica en la diseminación de las células cancerosas en órganos vitales, sin que sea algo exclusivo de tumores crecidos o avanzados, ya que estas pueden metastatizar desde estadios muy tempranos[48].

El proceso de metástasis en los carcinomas se puede esquematizar como una secuencia de pasos a menudo referidos como cascada metastásica y que abarca la invasión local por parte de las células tumorales, la intravasación a los vasos sanguíneos y linfáticos adyacentes, la circulación a través de los mismos, la extravasación al parénquima de tejidos distantes, la formación de pequeños nódulos de células tumorales (micrometástasis) y el crecimiento de estos nódulos hasta la completa colonización (macrometástasis)[49].

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[48] Massagué J. Evolución y metástasis del cáncer. Sociedad española de bioquímica y biología molecular. 2009;160.

[49] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

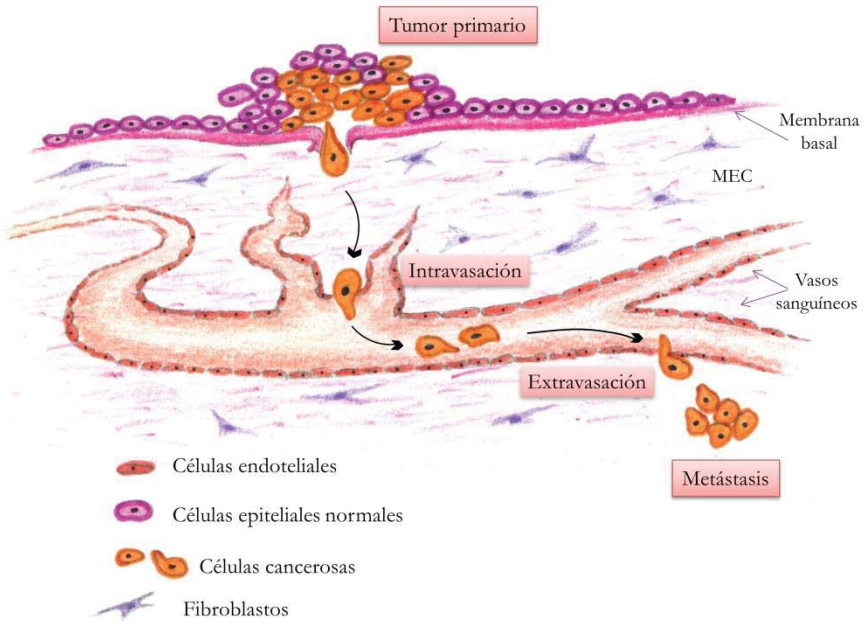

Figura 3. Cascada metastásica.

En un primer paso hacia la diseminación, por tanto, varias clases de proteínas involucradas en el amarre de las células a sus ambientes deberán estar alteradas en las células tumorales. Esto incluye moléculas de adhesión celular (CAMs) como inmunoglobulinas y familias de cadherinas (interacción célula-célula) e integrinas (interacción célula-matríz)[50]. Bajo estas condiciones, las células tumorales son capaces de migrar e invadir la membrana basal y la MET llevando a cabo la denominada transición epitelio-mesénquima (EMT), que consiste en la transformación de células epiteliales diferenciadas, organizadas y con

[50] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70.

polarización apico-basal a células semejantes a las mesenquimales. La degradación de la MET es crucial para abrirse paso y poder acceder a los sistemas vascular y linfático, lo que consiguen gracias a proteasas, principalmente MMPs, o empleando fuerzas mecánicas mediante la formación de protrusiones en el borde delantero de las células[51].

Una vez en circulación, además, necesitarán determinadas funciones para atravesar las paredes de los capilares del órgano a infiltrar, así como para sobrevivir en el nuevo entorno, donde la EMT será revertida del fenotipo mesenquimal al epitelial (transición mesénquima-epitelio)[52,51].

1.2. Cáncer de mama

1.2.1. Epidemiología

El cáncer de mama es el segundo tipo de cáncer más frecuente a nivel mundial, por detrás del cáncer de pulmón, estimándose 1,67 millones de nuevos casos (25% de todos los casos de cáncer) y 522.000 muertes (la quinta causa más común de muerte por cáncer) en 2012. En mujeres, sin embargo, es el tipo de cáncer con más incidencia, la primera causa de muerte por cáncer en regiones poco desarrolladas y

[51] Guan X. Cancer metastases: challenges and opportunities. Acta Pharm Sin B. 2015 Sep;5(5):402-18.

[52] Massagué J. Evolución y metástasis del cáncer. Sociedad española de bioquímica y biología molecular. 2009;160.

la segunda en regiones desarrolladas, por detrás nuevamente del cáncer de pulmón[53] (Figura 4).

En España, la incidencia de cáncer de mama fue de 25.215 mujeres en 2012, correspondiendo al 29% de los casos de cáncer entre el sexo femenino, y se estima que el número de afectadas ascenderá a 28.010 para el año 2020. Respecto a los datos de mortalidad, en 2012 se produjeron en nuestro país 6.075 defunciones por este tipo de cáncer, lo que representa el 15,5%, es decir, la más común entre las muertes causadas por cáncer en mujeres[54] (Figura 5).

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[53] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015 Mar 1;136(5):E359-86.

[54] International Agency for Research on Cancer (http://eco.iarc.fr/eucan).

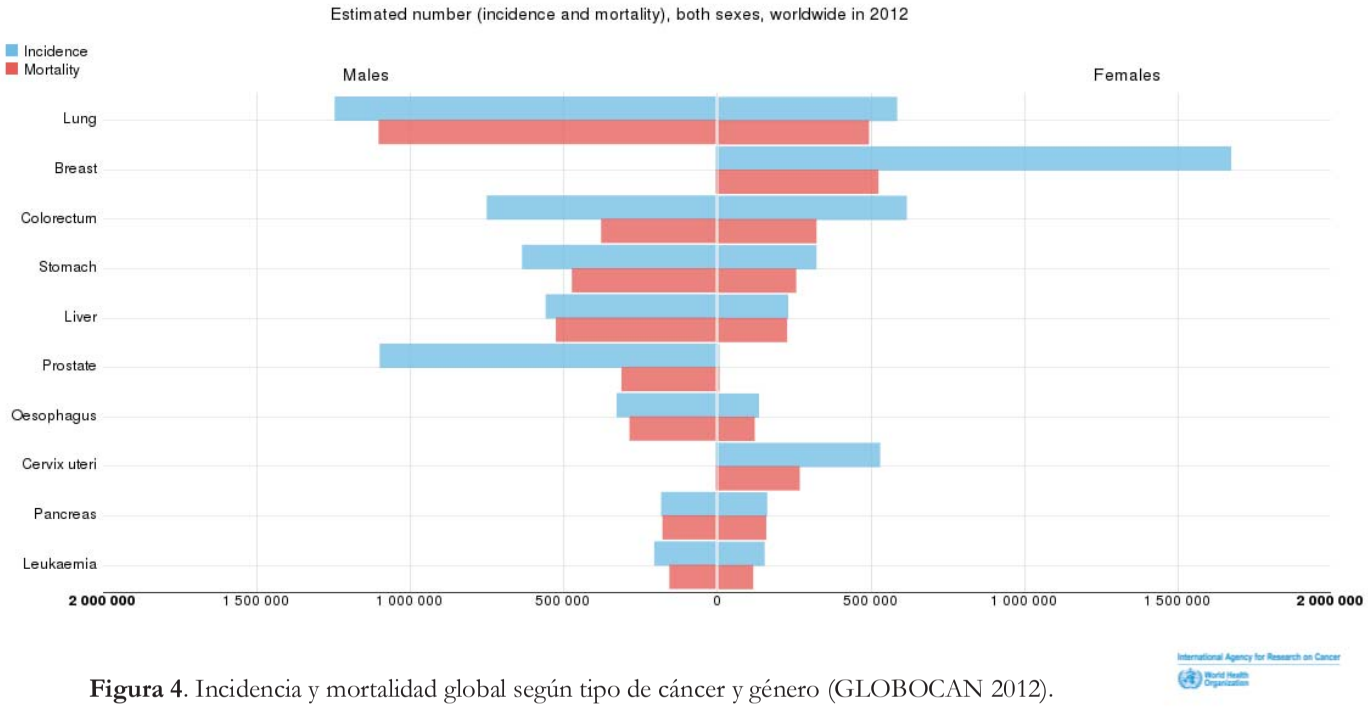

Figura 4. Incidencia y mortalidad global según tipo de cáncer y género (GLOBOCAN 2012).

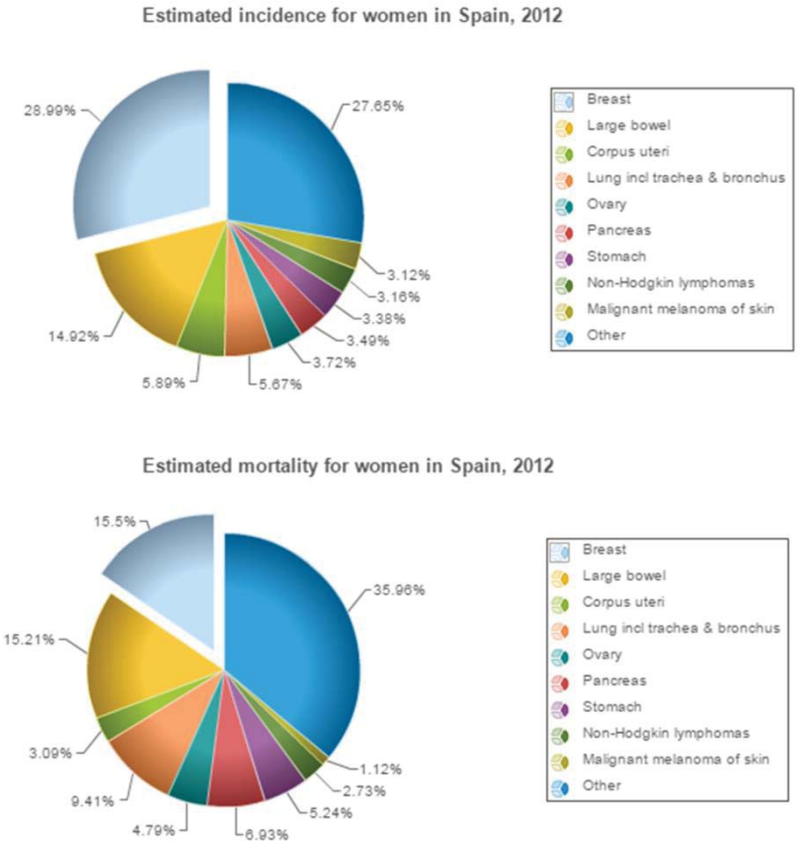

Figura 5. Incidencia y mortalidad en España según tipo de cáncer en mujeres (EUCAN 2012).

1.2.2. Particularidades

De los datos anteriores se deduce que el riesgo de padecer cáncer de mama es inherente a ser mujer, pero otros factores determinantes son el envejecimiento, una menarquia temprana, una menopausia tardía, la herencia genética y, sobre todo, factores epigenéticos. Solo entre el 5 y el 10% de los casos de cáncer de mama son hereditarios, siendo la

causa más común la mutación de los genes BRCA1 y BRCA2. En relación al estilo de vida, el riesgo de desarrollar este tipo de cáncer aumenta conforme a los siguientes factores[55]:

- consumo de bebidas alcohólicas
- sobrepeso u obesidad
- falta de actividad física
- nuliparidad o maternidad tardía (excepto para los tumores triple negativo, que definiremos a continuación)
- uso de anticonceptivos orales o inyectables
- uso de terapia de reemplazo hormonal

1.2.3. Clasificación de los tumores mamarios y su tratamiento

El cáncer de mama es una enfermedad muy heterogénea, y en su clasificación se siguen teniendo en cuenta los esquemas basados en la histología clásica, el tamaño del tumor, la invasión de los ganglios linfáticos y la diseminación metastásica, que en este caso puede ser a cuatro lugares: huesos, pulmones, hígado y cerebro, siendo los dos primeros los más frecuentemente afectados[56,57].

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[55] Kumar V, Abbas KA, Fausto N, Aster CJ. Robbins y Cotran. Patología estructural y funcional. 8ª ed. Elsevier; 2010.

[56] Massagué J. Evolución y metástasis del cáncer. Sociedad española de bioquímica y biología molecular. 2009;160.

[57] Taherian-Fard A, Srihari S, Ragan MA. Breast cancer classification: linking molecular mechanisms to disease prognosis. Brief Bioinform. 2015 May;16(3):461-74.

El estado de los ganglios linfáticos axilares, por ejemplo, es el factor pronóstico más importante en ausencia de metástasis distantes. En la mayoría de los carcinomas mamarios, los vasos linfáticos drenan primero en uno o dos ganglios (ganglios centinela), por lo que la biopsia de los mismos juega un papel crucial para la toma de decisiones clínicas[58].

Sin embargo, cada vez cobra más importancia la caracterización molecular como instrumento para comprender el pronóstico clínico, así como para predecir la respuesta a los tratamientos sistémicos. En este sentido es fundamental conocer el estado de los receptores de estrógenos (ER) y progesterona (PR), que reciben señales de crecimiento celular a través de la unión de estas hormonas, o del receptor 2 del factor de crecimiento epidérmico humano (HER2), que también estimula la proliferación de las células mediante la unión específica del factor de crecimiento epidérmico (EGF). Se conocen al menos dos tipos de ER, α y β, con diferentes funciones. El ERα se relaciona con el desarrollo y la progresión del cáncer, siendo al que nos referimos cuando hablamos de positividad para este receptor en cáncer de mama. El ERβ, por el contrario, se asocia con efectos antiproliferativos y proapoptóticos, papel que se ve reforzado por su baja expresión en células cancerosas de mama en comparación con células normales[59,60].

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[58] Kumar V, Abbas KA, Fausto N, Aster CJ. Robbins y Cotran. Patología estructural y funcional. 8ª ed. Elsevier; 2010.

[59] Haldosen LA, Zhao C, Dahlman-Wright K. Estrogen receptor beta in breast cancer. Mol Cell Endocrinol. 2014 Jan 25;382(1):665-72.

[60] Leygue E, Murphy LC. A bi-faceted role of estrogen receptor beta in breast cancer. Endocr Relat Cancer. 2013 May 30;20(3):R127-39.

Según esta clasificación molecular, se ha dividido el cáncer de mama en varios subgrupos con características biológicas y evoluciones clínicas distintivas[61,62]:

- Luminal A ($ER^+/PR^{+/-}/HER2^-$): los más frecuentes (40-55%). Presentan los mayores niveles de expresión de ER, por lo que responderán con mayor probabilidad al tratamiento hormonal, aunque no así a la quimioterapia estándar. Son de crecimiento lento y presentan un pronóstico favorable.
- Luminal B ($ER^+/PR^{+/-}/HER2^+$): el pronóstico es algo peor que el del subgrupo anterior, puesto que la tasa de proliferación es más elevada. Representan el 15-20% de los cánceres de mama.
- HER2 ($ER^-/PR^-/HER2^+$) (7-12%): presentan amplificación del gen HER2 y, con frecuencia, coamplificación y sobreexpresión de otros genes adyacentes. Con una tasa de proliferación elevada, el pronóstico era malo hasta la llegada del trastuzumab.
- Basal ($ER^-/PR^-/HER2^-$): suponen entre el 13-25% de los cánceres de mama y tienen un pronóstico desfavorable dada su elevada tasa de proliferación y metástasis, aunque a menudo responden a la quimioterapia. El término basal se debe a que expresan marcadores típicos de células consideradas basales, como las mioepiteliales y las progenitoras. Este subtipo se ha asociado a la mutación de BRCA1 y p53. A menudo se les denomina triple negativo, aunque este término no es preciso puesto que no todos los triples negativos expresan EGFR ni citoqueratinas 5/6, como ocurre en el subtipo basal.

[61] Kumar V, Abbas KA, Fausto N, Aster CJ. Robbins y Cotran. Patología estructural y funcional. 8ª ed. Elsevier; 2010.

[62] Casciato A, Territo C. Manual de oncología clínica. 7ª ed. LWW; 2013.

> Precisamente, ese porcentaje restante (6-10%) de triples negativos carece de una clasificación precisa, aunque algunos autores se refieren a ellos como subtipo normal, porque su perfil de expresión génica es similar al del epitelio mamario "normal".

En base a estas características se valorará, por tanto, el uso de alguno o varios tratamientos, los cuales incluyen cirugía (extirpación parcial o total del seno), radioterapia, quimioterapia, terapia hormonal y terapia dirigida.

La terapia hormonal se utiliza en cánceres ER y/o PR positivos y existen diferentes tipos. Los moduladores selectivos de los receptores de estrógeno (SERM), como el tamoxifeno, actúan bloqueando dichos receptores en las células mamarias y evitando así que el estrógeno se una a ellos, por lo que se usan principalmente en mujeres premenopáusicas. Este medicamento actúa como un estrógeno en otros tejidos que no son el de mama, a diferencia de medicamentos como el fulvestrant, que actúa como antiestrogénico en cualquier tejido y que por tanto se suele aplicar en cánceres metastásicos. También en mujeres premenopáusicas se usan los análogos de la hormona liberadora de hormona luteinizante, que suprimen la función de los ovarios e impiden que produzcan estrógeno. En mujeres posmenopáusicas, en las que los ovarios dejan de producir estrógenos, el medicamento de elección son los inhibidores de la aromatasa, que bloquean a esta enzima para que no se produzca estrógeno en el tejido adiposo.

Por otra parte, la terapia dirigida aprovecha alguna característica de las células cancerosas para combatirlas sin afectar a las células normales, como puede ser la amplificación de HER2. En este caso, el tratamiento más conocido es el trastuzumab, un anticuerpo

monoclonal humanizado con especificidad para el dominio extracelular de HER2[63].

Lamentablemente, los efectos secundarios de estos tratamientos son devastadores, por lo que cada vez con más frecuencia se buscan productos naturales que consigan minimizarlos siendo igualmente eficaces. A menudo, dichos productos podrían formar parte de la dieta, lo que nos lleva al siguiente apartado.

1.3. Nutrición y cáncer

Hoy en día, prácticamente todo el mundo sabe que la falta de algunos nutrientes es responsable de diversas enfermedades, como los casos de anemia y bocio ante el déficit de hierro y yodo. Lo que no parece estar tan arraigado en la conciencia social general es que el consumo de otros muchos "alimentos", o bien su exceso, repercute directamente en la salud y está asociado a un buen número de patologías. Así, los alimentos altamente procesados y refinados como aperitivos, cereales, bollería industrial, carnes, almidones, azúcares, ácidos grasos saturados (SFAs), grasas trans, etc., se relacionan con una mayor incidencia de ciertas enfermedades, como enfermedad cardiovascular (ECV), obesidad, síndrome metabólico, celiaquía, sensibilidad al gluten no celíaca y algunos tipos de cáncer[64,65]. Este es un aspecto a tener en

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[63] American Cancer Society. What are the risk factors for breast cancer?. 2016. Available from: http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-risk-factors.

[64] Trichopoulou A, Martinez-Gonzalez MA, Tong TY, Forouhi NG, Khandelwal S, Prabhakaran D, et al. Definitions and potential health benefits of the Mediterranean diet: views from experts around the world. BMC Med. 2014 Jul 24;12:112,7015-12-112.

cuenta puesto que, a veces, la clave no está en suprimir la ingesta de ciertos nutrientes sino en la cantidad y la calidad de los mismos. Un claro ejemplo de esto es lo que ocurre con los alimentos que contienen grasas. El aporte de grasa al organismo es necesario y no debe suprimirse, ya que esta proporciona energía y forma parte de estructuras como las membranas celulares. Sin embargo, la mayoría de los productos alimenticios industriales se preparan con grasas de origen animal, o de origen vegetal que han sido hidrogenadas (pudiendo producir grasas trans), con efectos adversos sobre la salud y que reemplazan a otras grasas beneficiosas como los ácidos grasos poliinsaturados (PUFAs) ω-3 de origen marino o el ácido oleico, un ácido graso monoinsaturado (MUFA). Este último predomina en el aceite de oliva virgen (AOV) que, a su vez, es la principal fuente de grasa en la dieta mediterránea.

1.3.1. La dieta mediterránea

La dieta mediterránea tradicional se caracteriza por el consumo elevado de hortalizas, legumbres, fruta, frutos secos, cereales no procesados y aceite de oliva virgen, junto al consumo moderado de productos lácteos, aves, pescado y vino en la comida, y al bajo consumo de dulces, carnes rojas y sus derivados. La Fundación Dieta Mediterránea va más allá del mero contexto nutricional y la expone como un estilo de vida que engloba también formas de cocinar, celebraciones, costumbres, productos típicos y actividades humanas diversas, lo que le ha permitido ser reconocida por la UNESCO como uno de los elementos de la Lista Representativa del Patrimonio Cultural Inmaterial de la Humanidad.

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[65] Mozaffarian D, Appel LJ, Van Horn L. Components of a cardioprotective diet: new insights. Circulation. 2011 Jun 21;123(24):2870-91.

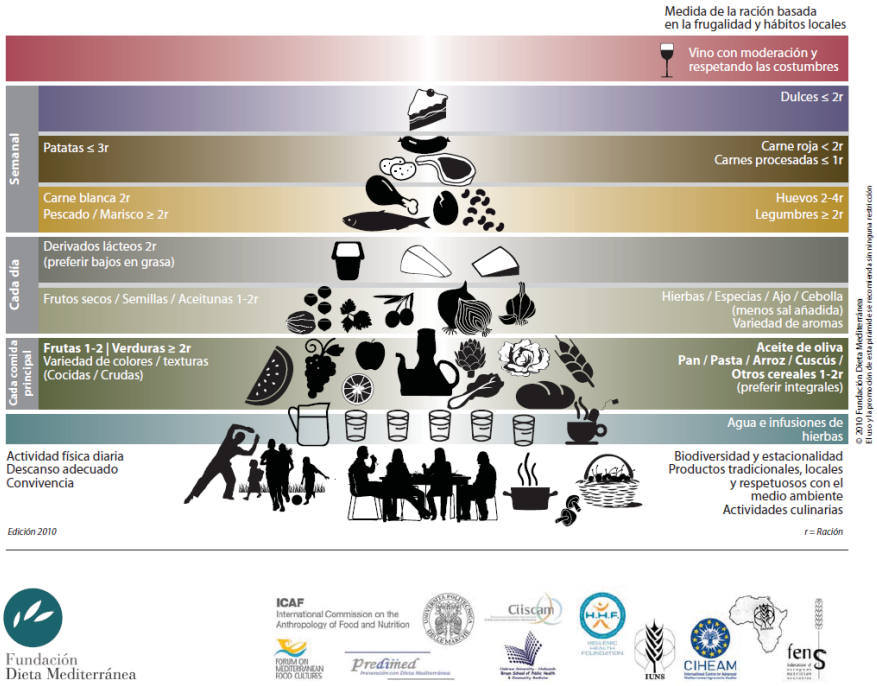

Figura 6. Pirámide de la Dieta Mediterránea. (Fundación Dieta Mediterránea)

Diferentes estudios han ido confirmando los beneficios de esta dieta, entre los que cabe mencionar la reducción en la incidencia de enfermedades cardiovasculares (incluyendo enfermedad coronaria, enfermedad arterial periférica, accidente cerebrovascular trombótico, etc.), diabetes tipo 2, síndrome metabólico, obesidad, deterioro cognitivo, depresión y cáncer, en especial el gástrico, el colorectal y el de mama[66,67,68,69,70,71]. En este sentido juegan un papel crucial los

[66] Trichopoulou A, Martinez-Gonzalez MA, Tong TY, Forouhi NG, Khandelwal S, Prabhakaran D, et al. Definitions and potential health benefits of the Mediterranean diet: views from experts around the world. BMC Med. 2014 Jul 24;12:112,7015-12-112.

estudios epidemiológicos de intervención clínica, siendo uno de los más relevantes el ensayo de prevención primaria cardiovascular PREDIMED (Prevención con Dieta Mediterránea), realizado en España entre 2003 y 2010[67]. Los resultados demuestran que una dieta mediterránea suplementada con AOV o frutos secos (y relativamente rica en grasa) usada en prevención primaria redujo en un 30% los eventos clínicos de ECV tras 5 años de intervención, en comparación con la recomendación de seguir una dieta baja en grasa. Actualmente, se está llevando a cabo un nuevo ensayo (PREDIMED-PLUS) en el que se intenta evaluar el efecto de una intervención intensiva sobre el estilo de vida en base a una dieta mediterránea tradicional con restricción de energía, actividad física y tratamiento conductual, sobre la incidencia de ECV y la pérdida de peso y su mantenimiento a largo plazo.

En el caso del cáncer de mama, recientes estudios epidemiológicos evidencian la relación entre la dieta mediterránea y un menor riesgo de padecer esta enfermedad, con especial reducción de tumores triple

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[67] Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med. 2013 Apr 4;368(14):1279-90.

[68] Salas-Salvado J, Bullo M, Estruch R, Ros E, Covas MI, Ibarrola-Jurado N, et al. Prevention of diabetes with Mediterranean diets: a subgroup analysis of a randomized trial. Ann Intern Med. 2014 Jan 7;160(1):1-10.

[69] Schroder H, Marrugat J, Vila J, Covas MI, Elosua R. Adherence to the traditional mediterranean diet is inversely associated with body mass index and obesity in a spanish population. J Nutr. 2004 Dec;134(12):3355-61.

[70] Couto E, Boffetta P, Lagiou P, Ferrari P, Buckland G, Overvad K, et al. Mediterranean dietary pattern and cancer risk in the EPIC cohort. Br J Cancer. 2011 Apr 26;104(9):1493-9.

[71] Gallus S, Bosetti C, La Vecchia C. Mediterranean diet and cancer risk. Eur J Cancer Prev. 2004 Oct;13(5):447-52.

negativo[72,73,74,75], lo que explicaría por qué la tasa de incidencia en zonas mediterráneas ha sido siempre menor que la de países del norte o centro de Europa y Estados Unidos.

Puesto que el AOV es la principal fuente de grasa de la dieta mediterránea y la principal diferencia respecto a otras dietas saludables, se cree que podría ser responsable de muchos de los efectos saludables atribuidos a la misma, lo que hace inevitable detenerse en sus características y propiedades.

1.3.2. Los aceites de oliva

El aceite de oliva virgen es el zumo oleoso de las aceitunas. Se trata de una grasa altamente energética, puesto que aporta unas 9 kcal/g, y que se mantiene líquida a temperatura ambiente gracias a su alto contenido en ácido oleico, lo que la diferencia de las grasas sólidas a temperatura

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[72] Toledo E, Salas-Salvado J, Donat-Vargas C, Buil-Cosiales P, Estruch R, Ros E, et al. Mediterranean Diet and Invasive Breast Cancer Risk Among Women at High Cardiovascular Risk in the PREDIMED Trial: A Randomized Clinical Trial. JAMA Intern Med. 2015 Sep 14;175(11):1752-60.

[73] Buckland G, Travier N, Cottet V, Gonzalez CA, Lujan-Barroso L, Agudo A, et al. Adherence to the mediterranean diet and risk of breast cancer in the European prospective investigation into cancer and nutrition cohort study. Int J Cancer. 2013 Jun 15;132(12):2918-27.

[74] Demetriou CA, Hadjisavvas A, Loizidou MA, Loucaides G, Neophytou I, Sieri S, et al. The mediterranean dietary pattern and breast cancer risk in Greek-Cypriot women: a case-control study. BMC Cancer. 2012 Mar 23;12:113,2407-12-113.

[75] Castello A, Pollan M, Buijsse B, Ruiz A, Casas AM, Baena-Canada JM, et al. Spanish Mediterranean diet and other dietary patterns and breast cancer risk: case-control EpiGEICAM study. Br J Cancer. 2014 Sep 23;111(7):1454-62.

ambiente, ricas en ácidos palmítico y esteárico (ambos SFAs), y menos digestibles[76].

Parece ser que el proceso de extracción del aceite de oliva virgen se remonta a las épocas paleolítica y neolítica en la zona de Creta. Desde allí, el cultivo del olivo y la obtención del aceite fue extendiéndose por toda la cuenca mediterránea, especialmente gracias a fenicios, griegos y romanos, no solo con fines alimenticios sino como agente balsámico, litúrgico, cosmético, higienizante y purificador[77].

Actualmente, a pesar de su expansión por todo el mundo, el cultivo del olivo y la producción de aceite siguen concentrándose en mayor medida en la zona mediterránea, donde se encuentran unas condiciones climáticas y edáficas favorables. En concreto, España es el mayor país productor y exportador de aceites de oliva a nivel mundial, según el Informe de noviembre de 2016 sobre producción y exportación de aceites de oliva del Consejo Oleícola Internacional (COI).

Tal y como refleja la Norma Comercial Aplicable a los Aceites de Oliva y los Aceites de Orujo de Oliva del COI (COI/T.15/NC nº3/Rev. 10. 2015)[78], se define a los aceites de oliva como los aceites procedentes únicamente del fruto del olivo (*Olea europea* L.), con exclusión de los aceites obtenidos por disolventes (lo que sería el aceite de orujo de oliva) o por procedimientos de reesterificación y de toda mezcla con aceites de otra naturaleza, existiendo las siguientes categorías:

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[76] Lozano-Sánchez J, Segura-Carretero A, Fernández-Gutiérrez A. Composición química del aceite de oliva. In: Fernández-Gutiérrez A, Segura-Carretero A, editors. El Aceite de Oliva Virgen: Tesoro de Andalucía. Málaga, España: Servicio de Publicaciones de la Fundación Unicaja; 2009. p. 195-224.

[77] Bartolini G, Petruccelli R. Origin, Diffusion and History of the Olive. Food and Agriculture Organization of the United Nations; 2002.

[78] Consejo oleícola Internaciónal (http://www.internationaloliveoil.org/).

- *Aceites de oliva vírgenes*: son los aceites obtenidos del fruto del olivo únicamente por procedimientos mecánicos o por otros medios físicos en condiciones, especialmente térmicas, que no produzcan la alteración del aceite, que no haya tenido más tratamiento que el lavado, la decantación, la centrifugación y el filtrado.

  Los aceites de oliva vírgenes aptos para el consumo en la forma en que se obtienen incluyen:

  - *Aceite de oliva virgen extra*: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 0.8 gramos por 100 gramos y cuyas demás características corresponden a las fijadas para esta categoría en la citada Norma.

  - *Aceite de oliva virgen*: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 2 gramos por 100 gramos y cuyas demás características corresponden a las fijadas para esta categoría en la citada Norma.

  - *Aceite de oliva virgen corriente*: aceite de oliva virgen cuya acidez libre expresada en ácido oleico es como máximo de 3.3 gramos por 100 gramos y cuyas demás características corresponden a las fijadas para esta categoría en la citada Norma.

  El aceite de oliva virgen no apto para el consumo en la forma en que se obtiene, denominado aceite de oliva virgen lampante, es el aceite de oliva virgen cuya acidez libre expresada en ácido oleico es superior a 3.3 gramos por 100 gramos y/o cuyas características organolépticas y demás características corresponden a las fijadas para esta categoría

en la citada Norma. Se destina a las industrias de refinado o a usos técnicos.

- *Aceites de oliva refinados*: son los aceites de oliva obtenidos de los aceites de oliva vírgenes mediante técnicas de refinado que no provoquen ninguna modificación de la estructura glicerídica inicial. Su acidez libre expresada en ácido oleico es como máximo de 0.3 gramos por 100 gramos y sus demás características corresponden a las fijadas para esta categoría en la citada Norma.

- *Aceites de oliva*: son los aceites constituidos por la mezcla de aceite de oliva refinado y de aceites de oliva vírgenes aptos para el consumo en la forma en que se obtienen. Su acidez libre expresada en ácido oleico es como máximo de 1 gramo por 100 gramos y sus demás características corresponden a las fijadas para esta categoría en la citada Norma.

La Unión Europea, sin embargo, excluye el concepto de aceite de oliva virgen corriente, y en su Reglamento de ejecución 2016/1227 relativo a las características de los aceites de oliva y de los aceites de orujo y sobre sus métodos de análisis[79], clasifica a los aceites de oliva vírgenes en función de la mediana de los defectos (media del defecto percibido con mayor intensidad) y de la mediana del atributo frutado, con los intervalos de referencia expuestos a continuación:

a) Aceite de oliva virgen extra: la mediana de los defectos es igual a 0 y la del atributo frutado es superior a 0.

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[79] Diario oficial de la Unión Europea. Reglamento de Ejecución (UE) 2016/1227 de la comisión de 27 de julio de 2016 relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis. (https://www.boe.es/doue/2016/202/L00007-00013.pdf).

b) Aceite de oliva virgen: la mediana de los defectos es superior a 0 pero inferior o igual a 3.5 y la del atributo frutado es superior a 0.

c) Aceite de oliva virgen lampante: la mediana de los defectos es superior a 3.5, o bien la mediana de los defectos es inferior o igual a 3.5 y la del atributo frutado es igual a 0.

1.3.2.1. Composición química del aceite de oliva virgen

A pesar de que la composición química exacta del AOV varía dependiendo de diversos factores como la variedad de aceituna, el grado de maduración de la misma, las condiciones climáticas, agronómicas y tecnológicas, etc., de forma general se distinguen una fracción mayoritaria o saponificable (98-99% del peso total del aceite) y una fracción minoritaria o no saponificable (2% del peso total del aceite) (Figura 7)[80].

[80] Lozano-Sánchez J, Segura-Carretero A, Fernández-Gutiérrez A. Composición química del aceite de oliva. In: Fernández-Gutiérrez A, Segura-Carretero A, editors. El Aceite de Oliva Virgen: Tesoro de Andalucía. Málaga, España: Servicio de Publicaciones de la Fundación Unicaja; 2009. p. 195-224.

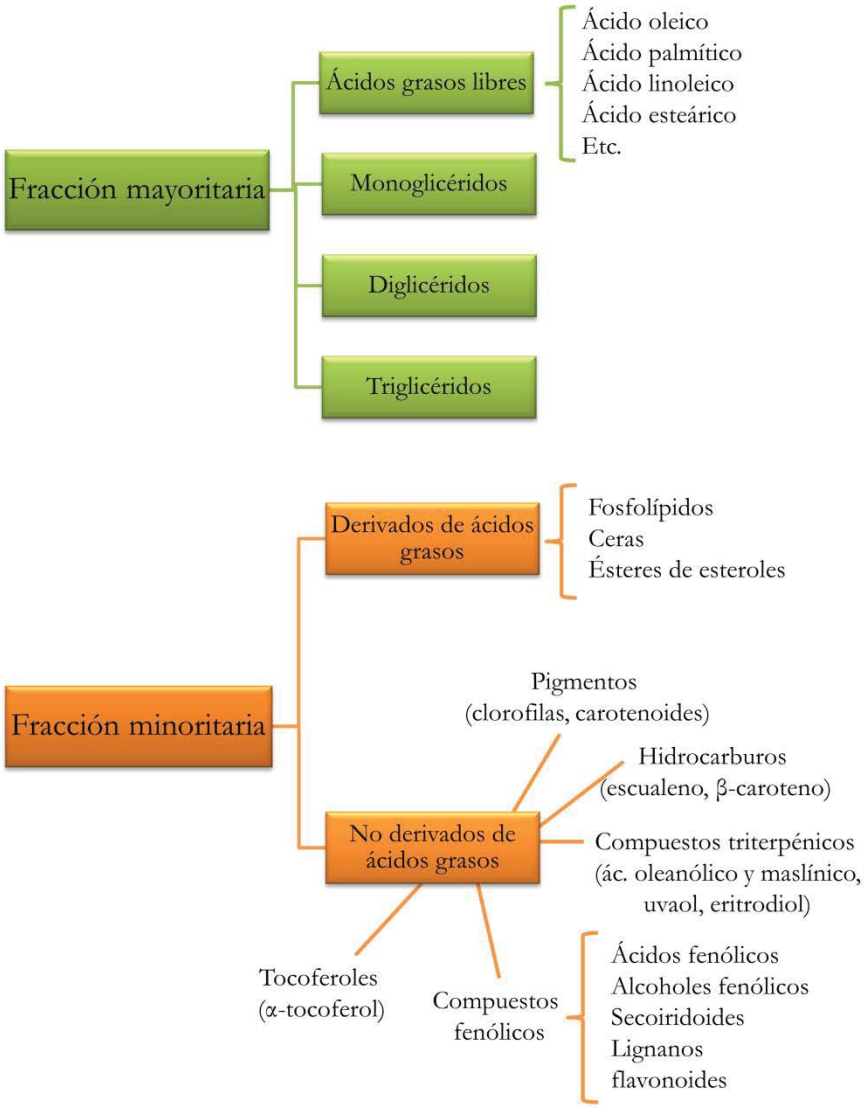

Figura 7. Composición química del AOV[81].

[81] Lozano-Sánchez J, Segura-Carretero A, Fernández-Gutiérrez A. Composición química del aceite de oliva. In: Fernández-Gutiérrez A, Segura-Carretero A, editors.

- Fracción mayoritaria:

Consta fundamentalmente de triglicéridos, que son ésteres compuestos por una molécula de glicerina y tres ácidos grasos, de entre los cuales el ácido oleico representa del 55 al 83%, convirtiendo al aceite de oliva en una grasa con un perfil monoinsaturado. Esto se traduce en una buena estabilidad frente a los radicales libres, en comparación con aceites que presentan mayormente PUFAs y que son más vulnerables a la oxidación. En mucha menor proporción se incluyen en esta fracción ácidos grasos libres, monoglicéridos y diglicéridos[82].

- Fracción minoritaria:

Se puede dividir a su vez en dos grupos, según sean o no derivados de ácidos grasos. Al primer grupo pertenecen los fosfolípidos, las ceras y los ésteres de esteroles, mientras que en el segundo grupo se pueden encontrar pigmentos (clorofilas, feofitinas y carotenoides), hidrocarburos (escualeno, β-caroteno, luteína), compuestos triterpénicos (ácido oleanólico, ácido maslínico, uvaol, eritrodiol), tocoferoles (α-tocoferol) y compuestos fenólicos.

De entre todos ellos, los compuestos fenólicos, junto con tocoferoles y carotenos, representan los antioxidantes más importantes del aceite de oliva, encontrándose las siguientes clases:

- Ácidos fenólicos: ácidos cafeico, gálico, vanílico, ferúlico, etc.
- Alcoholes fenólicos: tirosol e hidroxitirosol.

El Aceite de Oliva Virgen: Tesoro de Andalucía. Málaga, España: Servicio de Publicaciones de la Fundación Unicaja; 2009. p. 195-224.

[82] Lopez S, Bermudez B, Montserrat-de la Paz S, Jaramillo S, Varela LM, Ortega-Gomez A, et al. Membrane composition and dynamics: a target of bioactive virgin olive oil constituents. Biochim Biophys Acta. 2014 Jun;1838(6):1638-56.

- Secoiridoides: oleuropeína y ligustrósido.
- Lignanos: (+)-pinoresinol y 1-acetoxypinoresinol.
- Flavonoides: luteolina, apigenina y quercetina.

1.3.2.2. Propiedades bioactivas del aceite de oliva virgen

Cada vez son más numerosos los estudios que avalan que el AOV está detrás de la disminución en el riesgo de padecer ciertas patologías, como las enfermedades de tipo cardiovascular o el cáncer, principalmente de mama, colorectal y de próstata[83].

Además de sus demostradas propiedades como reductor de la hipertensión, se cree que los efectos antioxidantes del AOV podrían ser la causa de la baja incidencia de enfermedad coronaria asociada al consumo de la dieta mediterránea, ya que impidiendo la oxidación de colesterol LDL se evita la aparición de la aterosclerosis asociada a la misma[84]. A esta patología podría también contribuir positivamente el AOV a través de sus propiedades antiinflamatorias, ya que se ha descrito que reduce la IL6 y la proteína C reactiva, ambas asociadas a la aterosclerosis[85].

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[83] Lopez-Miranda J, Perez-Jimenez F, Ros E, De Caterina R, Badimon L, Covas MI, et al. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. Nutr Metab Cardiovasc Dis. 2010 May;20(4):284-94.

[84] Waterman E, Lockwood B. Active components and clinical applications of olive oil. Altern Med Rev. 2007 Dec;12(4):331-42.

[85] Fito M, Cladellas M, de la Torre R, Marti J, Munoz D, Schroder H, et al. Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. Eur J Clin Nutr. 2008 Apr;62(4):570-4.

En relación al cáncer, el carácter preventivo que se le atribuye podría estar mediado por cambios en la composición y la estructura de las membranas celulares o en las vías de señalización intracelular, la modulación de la expresión génica, del sistema inmunitario y del balance hormonal, y la reducción del estrés oxidativo y del daño al ADN[86].

Como ejemplo, el AOV es capaz de inducir apoptosis en células de cáncer colorectal y atenuar el inicio y la progresión de la carcinogénesis[81]. Diferentes trabajos también manifiestan la relación entre su consumo y la menor incidencia de cáncer de mama en humanos[87,88].

En comparación con el AOV, otras grasas como el aceite de maíz o el de girasol, así como las grasas saturadas, no solo no consiguen disminuir la carcinogénesis mamaria, sino que incluso podrían estimularla. Curiosamente, este efecto protector se ha observado también respecto a dietas bajas en grasa, lo que pone de manifiesto la importancia del tipo de aceite que se consume, por encima de su

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86 Escrich E, Solanas M, Moral R, Costa I, Grau L. Are the olive oil and other dietary lipids related to cancer? Experimental evidence. Clin Transl Oncol. 2006 Dec;8(12):868-83.

87 Toledo E, Salas-Salvado J, Donat-Vargas C, Buil-Cosiales P, Estruch R, Ros E, et al. Mediterranean Diet and Invasive Breast Cancer Risk Among Women at High Cardiovascular Risk in the PREDIMED Trial: A Randomized Clinical Trial. JAMA Intern Med. 2015 Sep 14;175(11):1752-60.

88 Trichopoulou A, Katsouyanni K, Stuver S, Tzala L, Gnardellis C, Rimm E, et al. Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. J Natl Cancer Inst. 1995 Jan 18;87(2):110-6.

contenido energético, y que la composición distintiva del AOV es fundamental a la hora de dar explicación a sus múltiples beneficios[89].

Gran parte de esos efectos beneficiosos del aceite de oliva se han atribuido tradicionalmente a su principal componente, el ácido oleico, del que se han descrito numerosas virtudes, muchas de ellas en relación a un menor riesgo de desarrollar enfermedades cardiovasculares, tales como la disminución de la presión sanguínea o la mejora del perfil lipídico y de la función endotelial, además de ser beneficioso frente a la diabetes tipo II mediante la producción de insulina y la inhibición de TNF-α, crucial en la inducción de esta enfermedad[90].

Este ácido graso se ha relacionado también con efectos beneficiosos en la respuesta inmunitaria, especialmente en enfermedades autoinmunes como artritis reumatoide o enfermedad inflamatoria intestinal, así como en la inmunidad asociada a funciones bactericidas y fungicidas.

Se ha demostrado que favorece la reparación del tejido dañado y el cierre de heridas a través del aumento en los niveles de colágeno III y la disminución de la enzima ciclooxigenasa-2 (COX-2), involucrada en la producción de mediadores proinflamatorios.

En el caso del cáncer, el ácido oleico parece tener un papel protector, sobre todo en cáncer de mama, ya que estudios *in vitro* han

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89 Sieri S, Chiodini P, Agnoli C, Pala V, Berrino F, Trichopoulou A, et al. Dietary fat intake and development of specific breast cancer subtypes. J Natl Cancer Inst. 2014 Apr 9;106(5):10.1093/jnci/dju068.

90 Sales-Campos H, Souza PR, Peghini BC, da Silva JS, Cardoso CR. An overview of the modulatory effects of oleic acid in health and disease. Mini Rev Med Chem. 2013 Feb;13(2):201-10.

demostrado que suprime la sobreexpresión del gen HER2, al mismo tiempo que actúa como coadyuvante de quimioterápicos como el trastuzumab, entre otros[91].

Cabe destacar que, puesto que se trata del zumo de la aceituna obtenido exclusivamente por medios mecánicos, el AOV mantiene muchos de los componentes biológicamente activos y relacionados con el aroma y la estabilidad del aceite, como son los compuestos fenólicos, que se pierden en el proceso de refinado del resto de aceites (incluido el aceite de oliva). Es en este punto donde los compuestos minoritarios cobran importancia, ya que varios estudios han demostrado mayor efectividad del AOV frente a, por ejemplo, la oxidación lipídica o la reducción de la inflamación en comparación con el aceite de oliva, que no posee muchos de estos componentes[92,93].

1.3.2.3. Propiedades bioactivas de los compuestos fenólicos

Los compuestos fenólicos presentes en el AOV contribuyen a la estabilidad del mismo y a su calidad sensorial, siendo responsables de

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[91] Menendez JA, Vellon L, Colomer R, Lupu R. Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu oncogene amplification. Ann Oncol. 2005 Mar;16(3):359-71.

[92] Covas M, Ruiz-Gutiérrez V, de la Torre R, Kafatos A, Lamuela-Raventós RM, Osada J, et al. Minor Components of Olive Oil: Evidence to Date of Health Benefits in Humans. Nutr Rev. 2006;64:S20-30.

[93] Fito M, Cladellas M, de la Torre R, Marti J, Munoz D, Schroder H, et al. Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. Eur J Clin Nutr. 2008 Apr;62(4):570-4.

características organolépticas como el picor o el amargor, no siempre bien recibidos por los consumidores, pero indicadores de alta calidad del aceite.

Muchos de los efectos saludables del AOV podrían atribuirse a su alto contenido en estos compuestos fenólicos, que como ya se ha comentado, son extraordinarios antioxidantes debido a la donación de electrones de su grupo hidroxilo, convirtiéndose en moléculas con mejor estabilidad y menor reactividad que otros antioxidantes[94,95,96]. De hecho, se ha confirmado que gracias a esta capacidad antioxidante, muchos de ellos logran prevenir las mutaciones y el desarrollo de cáncer propiciado por el daño de especies reactivas al ADN[97].

Estudios en diferentes líneas celulares de cáncer de colon han demostrado que los extractos fenólicos del AOV son capaces de reducir el daño al ADN, incrementar las funciones de barrera del epitelio y reducir la invasión de las células al tejido circundante,

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[94] Saeidnia S, Abdollahi M. Antioxidants: friends or foe in prevention or treatment of cancer: the debate of the century. Toxicol Appl Pharmacol. 2013 Aug 15;271(1):49-63.

[95] Toledo E, Salas-Salvado J, Donat-Vargas C, Buil-Cosiales P, Estruch R, Ros E, et al. Mediterranean Diet and Invasive Breast Cancer Risk Among Women at High Cardiovascular Risk in the PREDIMED Trial: A Randomized Clinical Trial. JAMA Intern Med. 2015 Sep 14;175(11):1752-60.

[96] Bendini A, Cerretani L, Carrasco-Pancorbo A, Gomez-Caravaca AM, Segura-Carretero A, Fernandez-Gutierrez A, et al. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules. 2007 Aug 6;12(8):1679-719.

[97] Escrich E, Solanas M, Moral R, Costa I, Grau L. Are the olive oil and other dietary lipids related to cancer? Experimental evidence. Clin Transl Oncol. 2006 Dec;8(12):868-83.

modulando por tanto, de manera beneficiosa, las etapas de inicio, progresión y metástasis del cáncer[98].

También en cáncer de mama han resultado ser beneficiosos los extractos fenólicos, como así indican trabajos con modelos de células tumorales en los que se inhibió la actividad de HER2 y la expresión de la enzima FASN, cuyos niveles suelen estar elevados en células tumorales[99,100].

Pero las cualidades de los compuestos fenólicos no solo se limitan a un efecto antitumoral, sino que abarcan también efectos antiinflamatorios, neuroprotectores o de retraso del envejecimiento. Algunos de estos compuestos ya cuentan con un amplio aval científico, como oleuropeina, hidroxitirosol, tirosol, escualeno, compuestos triterpénicos, etc.[101,102,103], mientras que otros aún están

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98 Gill CI, Boyd A, McDermott E, McCann M, Servili M, Selvaggini R, et al. Potential anti-cancer effects of virgin olive oil phenols on colorectal carcinogenesis models in vitro. Int J Cancer. 2005 Oct 20;117(1):1-7.

99 Menendez JA, Vazquez-Martin A, Garcia-Villalba R, Carrasco-Pancorbo A, Oliveras-Ferraros C, Fernandez-Gutierrez A, et al. tabAnti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial Extra-Virgin Olive Oil (EVOO). BMC Cancer. 2008 Dec 18;8:377.

100 Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, et al. Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med. 2008 Oct;22(4):433-9.

101 Warleta F, Quesada CS, Campos M, Allouche Y, Beltran G, Gaforio JJ. Hydroxytyrosol protects against oxidative DNA damage in human breast cells. Nutrients. 2011 Oct;3(10):839-57.

102 Warleta F, Campos M, Allouche Y, Sanchez-Quesada C, Ruiz-Mora J, Beltran G, et al. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Food Chem Toxicol. 2010 Apr;48(4):1092-100.

por conocer o se comienza poco a poco a descubrir sus cualidades, como es el caso de ciertos compuestos fenólicos, lignanos entre ellos.

Estos últimos han demostrado ser potentes antioxidantes *in vitro*, por delante del hidroxitirosol o derivados de la oleuropeína y el tirosol[104], por lo que representan compuestos a los que sería importante estudiar y conocer sus posibles propiedades biológicas.

1.3.2.4. Lignanos

El gran interés que suscita el grupo de los lignanos reside en que, además de compuestos fenólicos con una excelente capacidad antioxidante, son también fitoestrógenos, es decir, compuestos con estructura similar a la hormona estrógeno y que por tanto pueden actuar como moduladores hormonales a través de su acción estrogénica o antiestrogénica, con la consecuente importancia clínica en trastornos menopaúsicos o cáncer de mama.

Este grupo, uno de los más abundantes entre los compuestos fenólicos del AOV, incluye a los compuestos (+)-pinoresinol y 1-acetoxypinoresinol. A pesar de que la principal fuente de lignanos en el reino vegetal son las semillas de lino, el (+)-pinoresinol está presente en muy bajas concentraciones (la contribución de estas semillas a su ingesta media es solo del 0.3%)[105] y el 1-acetoxypinoresinol ni siquiera

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[103] Sanchez-Quesada C, Lopez-Biedma A, Warleta F, Campos M, Beltran G, Gaforio JJ. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of Olea europaea. J Agric Food Chem. 2013 Dec 18;61(50):12173-82.

[104] Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer. 2000 Jun;36(10):1235-47.

[105] Milder IE, Feskens EJ, Arts IC, Bueno de Mesquita HB, Hollman PC, Kromhout D. Intake of the plant lignans secoisolariciresinol, matairesinol,

ha sido identificado, siendo específico del olivo, las aceitunas y el AOV.

En el AOV fueron descritos, por primera vez, en el año 2000[106,107]. Owen *et al.* observaron concentraciones de hasta 100 mg/kg, aunque con variaciones considerables entre diferentes aceites, siendo el promedio total de lignanos de 41.53 ± 3.93 mg/kg. Brenes *et al.* encontraron diferencias de entre 11.7 a 41.2 mg/kg para el (+)-pinoresinol y 2.7 a 66.9 mg/kg para el 1-acetoxypinoresinol.

Estas variaciones, como ya hemos comentado, se deben a un gran número de variables, aunque en el caso de los lignanos existe cierta proporción para las diferentes variedades de aceitunas. Por ejemplo, el 1-acetoxypinoresinol es muy abundante en aceites Arbequina, Empeltre y Hojiblanca, mientras que su concentración es muy baja en los Picual, hecho que le ha situado como un indicador potencial para la autentificación de aceites de esta variedad[108,109].

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lariciresinol, and pinoresinol in Dutch men and women. J Nutr. 2005 May;135(5):1202-7.

106 Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer. 2000 Jun;36(10):1235-47.

107 Brenes M, Hidalgo F, Garcia A, Rios J, Garcia P, Zamora R, et al. Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. Journal of the American Oil Chemists' Society. 2000;77(7):715-20.

108 Brenes M, García A, Rios JJ, García P, Garrido A. Use of 1-acetoxypinoresinol to authenticate Picual olive oils. Int J Food Sci Tech. 2002;37(6):615-25.

109 Gomez Caravaca AM, Carrasco Pancorbo A, Canabate Diaz B, Segura Carretero A, Fernandez Gutierrez A. Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil. Electrophoresis. 2005 Sep;26(18):3538-51.

La estructura química de ambos compuestos es muy similar, excepto por la presencia de un grupo $–COOCH_3$ en el 1-acetoxypinoresinol. Constan de dos grupos fenólicos, cada uno de los cuales consiste en un anillo aromático unido a un grupo hidroxilo (OH) (Figura 8).

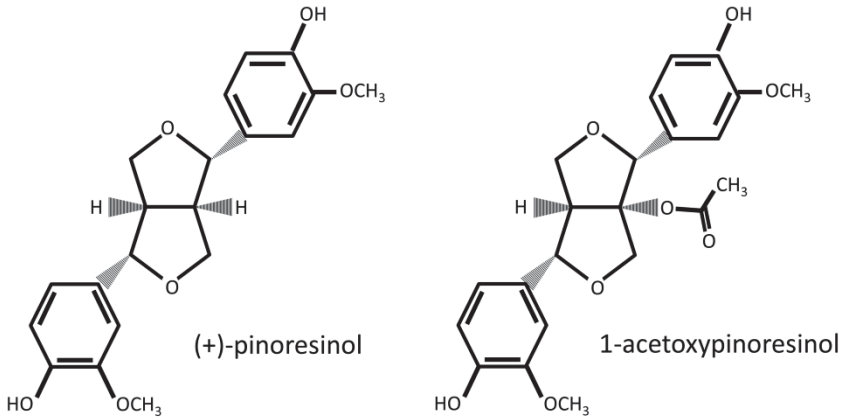

Figura 8. Estructura química de los lignanos (+)-pinoresinol y 1-acetoxypinoresinol.

Se ha relacionado a estos compuestos con diversas actividades biológicas tanto *in vitro* como *in vivo*. Aunque los trabajos experimentales en relación al 1-acetoxypinoresinol son muy escasos, debido fundamentalmente a que no está disponible comercialmente y a las dificultades en su aislamiento y síntesis, algunos estudios le atribuyen actividad captadora de radicales libres mayor que la de otros

antioxidantes como Trolox, hydroxytyrosol y oleuropeína[110] y efecto antitumoral en células cancerosas de mama SKBR3 y MCF7[111].

Las propiedades biológicas descritas para el (+)-pinoresinol incluyen actividad antioxidante, antiiflamatoria, hipoglucemiante, antifúngica, neuroprotectora y quimiopreventiva. Como antiinflamatorio, se ha descrito que inhibe la producción de NO y de mediadores proinflamatorios como TNFα, IL1β, IL-6, factor nuclear κβ (NF-κβ), prostaglandina E_2 (PGE_2) y COX-2. En relación a su efecto antitumoral, un estudio llevado a cabo en células de carcinoma de colon humano (HT115) mostró una disminución significativa de su capacidad de invasión[112], y un estudio caso-control encontró asociación entre el consumo elevado de (+)-pinoresinol y la reducción del riesgo de cáncer de mama entre mujeres premenopáusicas[113]. Además, diferentes trabajos muestran citotoxicidad en células tumorales humanas de mama, próstata, pulmón, ovario, piel y colon, aunque dicho efecto varía dependiendo de la línea celular utilizada y de

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[110] Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer. 2000 Jun;36(10):1235-47.

[111] Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, et al. Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med. 2008b Oct;22(4):433-9.

[112] Hashim YZ, Rowland IR, McGlynn H, Servili M, Selvaggini R, Taticchi A, et al. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. Int J Cancer. 2008 Feb 1;122(3):495-500.

[113] Torres-Sanchez L, Galvan-Portillo M, Wolff MS, Lopez-Carrillo L. Dietary consumption of phytochemicals and breast cancer risk in Mexican women. Public Health Nutr. 2009 Jun;12(6):825-31.

un estudio a otro, por lo que es difícil obtener conclusiones en este aspecto[114].

Cabe preguntarse si las concentraciones a las que los lignanos han mostrado estos efectos son las que se podrían encontrar en el organismo tras su consumo. Sin embargo, la ingesta solo se ha estimado para el (+)-pinoresinol en países no adheridos a la dieta mediterránea (Holanda, Canadá, Francia y Finlandia, con una media de 321 μg/d), por lo que el consumo debe ser mucho mayor en regiones donde el AOV forma parte de la alimentación diaria.

Por otra parte, el metabolismo de estos lignanos no está bien caracterizado y varía mucho entre individuos dependiendo de su dieta, la expresión de enzimas metabólicas o la microbiota intestinal. A pesar de que estos compuestos parecen mantenerse estables bajo las condiciones de digestión, tras la ingestión son parcialmente metabolizados a enterolignanos. Esta conversión fue del 55% en un modelo *in vitro* usando microflora humana[115], mientras que en células de colon humano se observó un metabolismo muy limitado[116].

De cualquier modo, parece ser que el (+)-pinoresinol se introduce en las células por difusión simple, sin necesidad de transportadores

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[114] López-Biedma A, Sánchez-Quesada C, Delgado-Rodríguez M, Gaforio JJ. The biological activities of natural lignans from olives and virgin olive oils: A review. Journal of Functional Foods. 2016;26:36-47.

[115] Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wahala K, Deyama T, et al. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem. 2001 Jul;49(7):3178-86.

[116] Soler A, Romero MP, Macià A, Saha S, Furniss CSM, Kroon PA, et al. Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. Food Chemistry. 2010;119:703-14.

específicos, por lo que estaría disponible para su absorción por cualquier tipo celular.

1.4. Justificación

En el repaso de las propiedades biológicas del AOV hemos visto cómo las evidencias científicas le otorgan un papel protector frente al desarrollo del cáncer de mama en humanos. Del mismo modo, los datos publicados sugieren que dicho efecto preventivo podría deberse en gran medida a la actividad de los compuestos minoritarios.

Partiendo de esta premisa, nuestro grupo de investigación viene desarrollando desde hace tiempo el estudio de algunos de estos compuestos minoritarios, arrojando luz acerca de la implicación que pudieran tener en la prevención y el desarrollo del cáncer de mama humano.

Dado que no se conocen los mecanismos moleculares que podrían verse modificados por la acción de estos compuestos, los ensayos se han llevado a cabo sobre modelos celulares, como un primer paso para conocer los efectos que pudieran tener sobre el organismo y como base para posteriores estudios en modelos animales y ensayos clínicos.

Los trabajos realizados con modelos experimentales celulares permiten el estudio del comportamiento de las células libres de las variaciones que tienen lugar dentro de un organismo y bajo el estrés de un experimento, aumentando el conocimiento de los mecanismos biológicos básicos y permitiendo la reproducibilidad del método científico. Por ello, resulta imprescindible que la búsqueda de nuevos tratamientos comience en el laboratorio, como base para posibles ensayos *in vivo* o investigaciones clínicas posteriores.

De hecho, la Autoridad Europea de Seguridad Alimentaria (EFSA, European Food Safety Authority) incluye los estudios *in vitro* como parte de las evidencias que han de tenerse en cuenta en la obtención de alegaciones de salud de alimentos o componentes alimenticios, y la UE financia proyectos de investigación con ensayos *in vitro* basados en líneas celulares humanas, destinados a comprender los efectos y las rutas metabólicas de fármacos o componentes químicos cuya toxicidad se desconoce y que por tanto no pueden ser susceptibles de análisis mediante la exposición a humanos.

Desde dicho enfoque, hemos estudiado las propiedades de los compuestos minoritarios más abundantes, pero cuyos efectos en cáncer de mama no estaban descritos, como el hidroxitirosol, el tirosol, el escualeno, el ácido oleanólico, el ácido maslínico, el uvaol y el eritrodiol, lo que ha dado lugar a diferentes trabajos que han servido además para la lectura de anteriores tesis doctorales. Así, por ejemplo, hemos comprobado *in vitro* la acción preventiva frente al cáncer de mama de los fenoles simples hidroxitirosol y tirosol y del hidrocarburo escualeno, a través de su capacidad antioxidante intracelular, así como la ausencia de capacidad antitumoral para estos compuestos. El carácter quimiopreventivo obtenido mediante la disminución del estrés oxidativo y el daño al ADN en células no tumorales de mama humana también se ha descrito para el ácido oleanólico, que además es capaz de inhibir la proliferación y producir, al igual que el ácido maslínico y el eritrodiol, daño al ADN de células tumorales de mama altamente metastásicas. Hemos descrito además la capacidad del ácido maslínico para prevenir la inflamación crónica, estrechamente relacionada con el desarrollo de cáncer, a través de la modulación de la respuesta inflamatoria y promoviendo la diferenciación de los macrófagos al estado M1.

En conjunto, estos resultados sugieren la participación de los componentes minoritarios, en mayor o menor grado, en los efectos

beneficiosos del consumo de AOV en la prevención de la incidencia de cáncer de mama.

Siguiendo esta línea, el compuesto (+)-pinoresinol llamó nuestra atención por su importancia cuantitativa y sus peculiaridades biológicas, ya que tanto su capacidad antioxidante como compuesto fenólico, esencial en la prevención frente al desarrollo de cáncer, como su carácter fitoestrogénico, clave en el caso de tumores mamarios, podría ser útil en la terapia frente al cáncer y/o ser responsable del efecto preventivo frente al cáncer de mama que se asocia al consumo de AOV en el contexto de una dieta mediterránea.

Estos hechos fundamentan los estudios llevados a cabo en la presente tesis, que abarcan el análisis de los efectos del (+)-pinoresinol como posible agente antioxidante, antitumoral y quimiopreventivo, en diferentes modelos experimentales de células de mama humanas.

2. Hipótesis y objetivos

Hipótesis

Considerando que:

1. los estrógenos juegan un papel importante tanto en la génesis como en la evolución del cáncer de mama,
2. los aceites de oliva vírgenes contienen fitoestrógenos, siendo el más importante de ellos el (+)-pinoresinol,
3. el consumo habitual de aceites de oliva vírgenes se asocia a una menor incidencia de cáncer de mama,

nuestra hipótesis es que: el (+)-pinoresinol podría ser responsable, al menos parcialmente, de esta última evidencia gracias a sus propiedades antitumorales y/o quimiopreventivas.

Objetivo general

Dilucidar los posibles efectos quimiopreventivos y/o antitumorales que el lignano (+)-pinoresinol pudiera tener sobre un modelo experimental tumoral *in vitro*, utilizando células mamarias humanas con diferentes patrones de expresión de receptores hormonales, dado el carácter fitoestrogénico de este compuesto.

Objetivos parciales

- Estudiar la capacidad antioxidante química del (+)-pinoresinol.

- Evaluar el posible efecto antitumoral del (+)-pinoresinol en las células tumorales de mama humanas MCF-7, no metastásicas y positivas para los receptores de estrógeno y progesterona, en base a: su actividad citotóxica, antiproliferativa, inducción de apoptosis, alteración del ciclo celular, efecto sobre los niveles de EROs y, sobre el daño al ADN.

- Evaluar el posible efecto antitumoral del (+)-pinoresinol en las células tumorales de mama humanas MDA-MB-231, altamente metastásicas y negativas para los receptores de estrógeno y progesterona, en base a: su actividad citotóxica, antiproliferativa, inducción de apoptosis, alteración del ciclo celular, efecto sobre los niveles de EROs y, sobre el daño al ADN.

- Determinar el posible efecto quimiopreventivo del (+)-pinoresinol en las células epiteliales de mama humanas

MCF10A, evaluando su actividad citotóxica; sobre la proliferación celular; inducción de apoptosis; efecto sobre el ciclo celular; estrés oxidativo y; protección frente al daño al ADN.

3. Trabajo experimental

Con el propósito de facilitar la lectura, en este apartado se exponen los trabajos publicados en el mismo formato utilizado hasta ahora, encontrándose los formatos originales en el apartado "ANEXOS".

3.1. Artículo 1

Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses.

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Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses.

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Abstract

• Background: Consumption of virgin olive oil (VOO) has been associated with a low breast cancer incidence. Pinoresinol is a phytoestrogen that is typically found in VOO. Considering the role of oestrogen in breast cancer development and progression, we investigated the potential antitumor activity of pinoresinol in breast cancer cells.

• Methods: To address this question, we treated MDA-MB-231 (oestrogen receptor [ER] negative) and MCF7 (ER+) human breast

tumour cells and MCF10A human mammary epithelial cells (ER-) with different concentrations of pinoresinol. The cytotoxic activity, cell proliferation, cell cycle profile, apoptosis induction, reactive oxygen species production and DNA damage were assessed.

• Results: Pinoresinol showed cytotoxic, anti-proliferative and pro-oxidant activity in human breast tumour cells, independent of their oestrogen receptor status. In addition, pinoresinol exerted antioxidant activity and prevented DNA damage associated with oxidative stress in human mammary epithelial cells.

• Conclusions: Overall, the results suggest that pinoresinol may have antitumor activity in human breast cancer cells independently of oestrogen receptor status. Furthermore, the results show that the pinoresinol has the typical characteristics of a chemopreventive compound.

Keywords: Cytotoxic activity; Antioxidant; Virgin olive oil; Polyphenols; Chemopreventive

Background

Growing scientific evidence suggests that the intake of virgin olive oil (VOO), which is the main source of fat in Mediterranean diets, correlates with a low incidence of breast cancer [1]. Among the minor compounds present in VOO that possess different health properties [2-6], we find polyphenols to be a very interesting group because of their biological benefits. It has been reported that polyphenols prevent the development and progression of pathological conditions, such as cancer, neurological and cardio-vascular diseases, diabetes, aging, and so on [7].

One of the most abundant phenolic compounds in VOOs, behind tyrosol and hydroxytyrosol, is (+)-pinoresinol (PINO) [8] (Figure 1). Its presence in VOOs depend on the variety of the cultivar, in amounts of 0.07 ± 0.003 mg/kg in Arbequina variety, about 0.90 ± 0.78 in Picual variety [9]. Brenes et al. [10] reported that Spanish olive oil contains a range of 20 to 45 mg/kg PINO. Several health properties have been attributed to PINO, including antifungal [11], anti-inflammatory [12, 13], hypoglycaemic [14] or chemopreventive biological activities [15, 16].

PINO has a chemical structure that is similar to that of oestrogen (i.e., it is a phytoestrogen). Oestrogen is essential for the growth and development of mammary glands and has been linked with the development and progression of breast cancer due to enhanced binding and activation of the oestrogen receptor α (ERα) [17]. For example, the phytoestrogen tamoxifen acts as an oestrogen antagonist in breast tissue and has been shown to slow breast cancer cell proliferation and has been used in clinical practice for breast cancer patients [18].

Interestingly, ERβ has also been shown to mediate estrogenic action. The specific role of this receptor in human breast cancer remains elusive; however, in contrast to ERα, ERβ has been linked with anti-proliferative and pro-apoptotic activities. In fact, the expression of ERβ is lower in human breast cancer cells compared to normal breast cells, supporting its potential tumour-suppressive role [19].

Surprisingly, very few studies have noted the role of PINO as a potential agonist or antagonist of oestrogen and the chemopreventive repercussions that PINO treatment may have on hormone-related breast cancer [20].

The chemical antioxidant activity of PINO also remains unclear. A few studies using DPPH and ABTS assays have shown different antioxidant functions of PINO [21-23]. However, the Oxygen Radical

Absorbance Capacity method (ORAC) has not been used in past studies, despite being considered one of the most biologically relevant assays [24].

Furthermore, the little research that has been done surrounding the effects of this compound on breast cancer cells remains inconclusive. Chin et al. [21] described a lack of cytotoxic effects and a cytoprotective effect of PINO on MCF7 cells stressed by H2O2 [25]. Other authors have reported anticancer effects of PINO by suppressing the expression of the lipogenic enzyme FASN in HER-2 overexpressing MCF7 cells [26]. Recently, Sepporta et al. [27] observed that PINO inhibited the growth of MDA-MB-231 cells, but not of MCF7 cells. Importantly, no previous study has examined the effects of PINO on a normal human breast cell line, which would address whether PINO plays a protective role against cancer development.

Therefore, the aim of the present study was to examine whether PINO exerts chemopreventive and/or antitumor activity in breast cancer, specifically because this compound is found in VOO and its consumption has been related with a minor incidence of breast cancer.

Therefore, to determine whether this compound may contribute, at least in part, to the health benefits attributed to VOO on breast cancer incidence and mortality, we studied the effects of PINO on breast cells with different receptor expression patterns. For this purpose, we used the following human mammary cells: highly invasive MDA-MB-231 (oestrogen receptor [ER] and progesterone receptor [PR] negative) breast tumour cells, the minimally invasive MCF7 (ER and PR positive) breast tumour cells and MCF10A human mammary epithelial cells (ER and PR negative).

Methods

Chemicals and material

The following were purchased from Gibco® Life Technologies Ltd (Paisley, UK): HuMEC Ready Medium (1X), TrypLE™ Express Enzyme (1X) and Minimum Essential Medium (MEM). Foetal bovine serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, Austria). Ethanol 96% v/v and potassium peroxodisulfate ($K_2S_2O_8$) (CAS 7727-21-1) were purchased from Panreac Química S.L.U. (Barcelona, Spain). The CellTiter-Blue® Cell Viability Assay was acquired from Promega Corporation (Madison, WI, USA). Round bottom culture plates and cell culture flasks were purchased from Nunc A/S (Roskilde, Denmark). Flat bottom culture plates were from CytoOne (Hamburg, Germany). Fluorescein (FL) (CAS 2321-07-5) was obtained from Life Technologies (Carlsbad, CA, USA). The following were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA): (+)-Pinoresinol (CAS 487-36-5) purity ≥95%; PBS; (S)-(+)-camptothecin (CPT) (CAS 7689-03-4) purity ≥90%; 2´,7´-dichlorofluorescin diacetate (DCFH-DA) (CAS 4091-99-0) purity ≥97%; Sodium pyruvate solution (CAS 113-24-6); MEM Non-essential Amino Acid Solution (NEAA); HEPES buffer solution (CAS 7365-45-9); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (CAS 1898-66-4); 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (CAS 30931-67-0) purity ≥98%; (±)-α-Tocopherol (Vitamin E) (CAS 10191-41-0) purity ≥96%; (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox(TM)) (CAS 53188-07-1) purity 97% and 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) (CAS 2997-92-4) purity 97%. PBS (1X, Dulbecco's) and DMSO (CAS 67-68-5) were obtained from AppliChem GmbH (Darmstadt, Germany). The PI/RNase Staining Buffer kit, Annexin V-FITC kit and Comet Assay kit (CAS 50-07-7) were purchased, respectively, from BD Biosciences, Pharmingen (San Diego, CA), Miltenyi Biotec (Bergisch Gladbach, Germany) and

Trevigen, Inc. (Gaithersburg, MD, USA). Non-tumorigenic human breast epithelial cells (MCF10A), minimally invasive human breast cancer cells (MCF7) and highly invasive human breast cancer cells (MDA-MB-231) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

ABTS radical scavenging test

The ABTS radical scavenging activity was measured as previously reported [28]. ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting 7 mM ABTS with 2.45 mM $K_2S_2O_8$ (final concentration) for 16 h in the dark at room temperature. The radical obtained was diluted in ultrapure water until the absorbance at 734 nm was between 0.7 and 1. Ethanol solutions of Trolox™ (antioxidant standard) or pinoresinol (PINO) were diluted in ultrapure water to reach concentrations between 50 and 800 μM and 0.00001 and 1000 μM, respectively. Twenty microliters of each concentration of Trolox™, PINO, ultrapure water (blank) or ethanol control (10%) were added into a flat bottom 96-well plate. The reaction was initiated by the addition of 50 μL of $ABTS^{\bullet+}$, and the absorbance at 734 nm was immediately measured every 5 min over 2 h at 30oC with a TECAN GENios Plus microplate reader (Tecan Group Ltd., Switzerland). All of the reactions were performed in triplicate, in three independent experiments. The percentage of the radical scavenging activity (% RSA) was calculated according to the following formula:

$$\% \text{ RSA} = 100 \, (A_{C(0)} - A_{A(t)})/A_{C(0)} \quad (1)$$

where $A_{C(0)}$ is the absorbance of the blank at t = 0 and $A_{A(t)}$ is the absorbance of the compound/standard at t = 60.

Radical scavenging activity by the DPPH assay

Estimation of the antioxidant capacity against the radical DPPH was carried out according to Brand-William et al., [29] with some modifications. An ethanolic solution of DPPH 100 μM (final concentration) was mixed in 96-well plates with ethanolic solutions of PINO or α-tocopherol (antioxidant standard) at 0.03, 0.06, 0.13, 0.25, 0.5, 1 and 2 mole ratios (moles of antioxidant/moles of DPPH). DPPH samples without antioxidants were also measured as blank controls. The absorbance at 520 nm was read every 5 min over 2 h with a TECAN GENios Plus microplate reader. Measurements were performed at least in triplicate in three separate experiments. The radical scavenging activity (% RSA) was calculated as described in Eq. (1) (t=60).

$ORAC_{FL}$ assay

The Oxygen Radical Absorbance Capacity (ORAC) of PINO was assayed as described in Prior et al. [30]. This method measures the oxidative degradation of fluorescein induced by the thermal decomposition of the AAPH azo-compound. In brief, fluorescein (48 nM) was added to each well of a round bottom 96-well plate that was previously tempered at 37°C. Then, PINO (from 0.001 to 1000 μM), Trolox™ (standard, from 12.5 to 100 μM) or PBS (blank) with a final volume of 1% DMSO (v/v) was added to the wells. After incubating for 15 min at 37°C, AAPH was added to the wells. Fluorescence readings (Ex. λ_{485}/Em. λ_{520} nm) were taken every 5 min at 37°C for 160 min with a TECAN GENios Plus microplate reader. The 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 determined as follows:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_n/f_0 \quad (2)$$

where f_0 is the initial fluorescence at cycle 0 and f_n is the fluorescence reading at cycle *n*.

The results were expressed as micromolar Trolox™ equivalents (TE), which were calculated using the line equation from the standard curve:

$$TE = (Y - b)/m \quad (3)$$

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

Cell culture and treatments

Human MCF10A (ERα and PR negative) breast epithelial cells were grown in HuMEC Ready Medium. Human MCF7 (ERα and PR positive) and MDA-MB-231 (ERα and PR negative) breast cancer cells were grown in MEM supplemented with 10% FBS, 1% Hepes buffer, 1% NEAA and 1% Sodium Pyruvate. The cells were cultivated as monolayer cultures in a humidified atmosphere with 5% CO_2 at 37°C and subcultured using TryPLE Express. Cells growing between 90 and 95% of confluence were used for all experiments. The cells were treated for 24 h with 0.001, 0.01, 0.1, 1, 10 and 100 μM of PINO that was previously dissolved in DMSO (stock concentration 50 mM).

Cytotoxicity Assay

The effects of PINO on cell viability were determined by the CellTiter-Blue® Cell Viability Assay according to the manufacturer's protocol with some modifications. A total of $5x10^3$ cells/well (for MDA-MB-231 and MCF7) or $2.5x10^3$ cells/well (for MCF10A) were seeded onto a 96-well plate. After 24 h to allow for cell attachment, the cells were treated with PINO or DMSO (as vehicle control) for another 24 h. CellTiter-Blue® was then added, and the plates were

incubated for 3 h in darkness at 5% CO_2 and 37ºC. Finally, fluorescence was read with a TECAN GENios Plus microplate reader (Ex. λ_{485}/Em. λ_{595} nm) and viability was calculated using the formula:

$$\% \text{ viable cells} = [(A_{\text{treated cells}}) / (A_{\text{control}})] \times 100 \quad (4)$$

where A corresponds to the relative fluorescence units of each sample. All of the measurements were performed in triplicate and each experiment was repeated at least three independent times.

Cell proliferation assay

In all of the cell proliferation experiments performed, the cells were seeded cells onto 96-well plates and allowed to attach before adding PINO or DMSO as the vehicle control. After 24 h of treatments, the medium was replaced by fresh medium and the plates were incubated for another 24 h. Then, CellTiter-Blue® was added, and fluorescence was read after 3 h of incubation with a TECAN GENios Plus microplate reader (Ex. λ_{485}/Em. λ_{595} nm). The measurements were repeated at 48, 72 and 96 h. The percentage of viable cells was calculated as defined in Eq. (4).

Cell cycle analysis

A total of 1 x 10^5 cells/mL (for MDA-MB-231 and MCF7 cells) or 5 x 10^4 cells/mL (for MCF10A cells) were seeded and allowed to attach for 24 h before treating with PINO for another 24 h. The cells were then fixed in cold 70% ethanol, stored at -20ºC for at least 24 h and labelled with a PI/RNase Staining Buffer kit. Cell cycle assessment was conducted by flow cytometry in an EPICS XL-MLC flow cytometer (Beckman Coulter, Spain), and the results were analysed

using the FlowJo program (v5.7.2). Each experiment was repeated three independent times.

Apoptosis analysis

MDA-MB-231 (1 x 10^5 cells/mL), MCF7 (1 x 10^5 cells/mL) or MCF10A (5 x 10^4 cells/mL) cells were seeded, allowed to attach and treated for 24 h with PINO. The cells and supernatants were collected and labelled with Annexin V-FITC kit according to the manufacturer's suggestions. As a positive control, the cells were incubated with 1 μM camptothecin (CPT). Apoptosis analysis was carried out using an EPICS XL-MLC flow cytometer, and the results were analysed using the FlowJo program. Each experiment was repeated three independent times.

Detection of reactive oxygen species

Detection of intracellular Reactive Oxygen Species (ROS) was performed using the probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as previously reported by our group [31]. In brief, MCF10A ($5.5x10^3$ cells/well), MDA-MB-231 or MCF7 cells ($7x10^3$ cells/well) were seeded onto 96-well plates, allowed to attach for 24 h and then treated with PINO for an additional 24 h. After the addition of DCFH-DA (100 μM), the plates were incubated for 30 min at 37°C and 5% CO_2. Fluorescence was then read for 30 min (Ex. λ_{485}/Em. λ_{535}) with a TECAN GENios Plus microplate reader.

It is well known that the addition of H_2O_2 increases stress in culture cells [32]. To test whether PINO had a protective role against induced oxidative stress, the assay was also performed after the addition of H_2O_2 (400 μM) 30 min before quantification.

Both experimental conditions were assayed three independent times, and each measurement was performed in quadruplicate. In all cases, iron free media (MEM or HuMEC) were used.

The intracellular ROS level percentage was calculated as follows:

$$F = [(Ft_{30} - Ft_0)/Ft_0] \times 100 \quad (5)$$

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

Alkaline single-cell gel electrophoresis (Comet assay)

To estimate the state and wholeness of DNA, $5x10^4$ cells/well (for MCF10A cells) or $1x10^5$ cells/well (for MCF7 and MDA-MB-231 cells) were allowed to attach to a 12-well plate and treated with increasing PINO concentrations for 24 h. The cells were then detached and centrifuged twice in PBS. To evaluate whether PINO had the ability to protect against oxidative DNA damage, cells were also exposed to H_2O_2. The comet assay was carried out according to Warleta et al. [3]. Analysis of the DNA strands was performed by examining twenty-five random cell images per sample in a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss; Jena, Germany) equipped with Luca EMCCD camera (Andor Technology, Belfast, UK) and using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). DNA damage was calculated by determining the relative fluorescence between the head and tail using the olive tail moment (Olive_TM), which was defined as:

$$Olive\_TM = [(tail\ (mean) - head\ (mean)) \times tail\ (\%\ DNA)]/100 \quad (6)$$

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test with the STATGRAPHICS Centurion XVI software (Statpoint Technologies, Inc. Warrenton, VA, USA). The values of $p<0.05$ were considered significant. The data are represented as the mean of at least three independent experiments ± SEM and are expressed relative to the untreated controls.

Results

ABTS radical scavenging test

The radical cation $ABTS^{\bullet+}$ was diminished by pinoresinol (PINO) above 10 μM (Figure 2), and the studied range was from 0.00001 to 1000 μM. Concentrations lower than 10 μM did not shown antioxidant capacity (data not shown). The antioxidant effects of PINO were higher than the antioxidant standard, as the 50% of Radical Scavenging Activity (RSA) occurred at 380 μM for Trolox™ and at 274 μM for PINO.

Radical scavenging activity by the DPPH assay

As depicted in Figure 3, PINO exhibited antioxidant activity against the DPPH radical in a dose dependent manner. The RSA of PINO was determined to be 50% at 0.69 mol ratio (69 μM), while the RSA of the antioxidant control α-tocopherol was 50% at 0.11 mol ratio (11 μM).

$ORAC_{FL}$ Assay

The peroxyl radical scavenging activity of PINO, as measured by $ORAC_{FL}$, showed a protective effect against AAPH-induced peroxyl radical activity. PINO exerted a higher protection than Trolox™. The micromolar Trolox™ equivalents (TE) values were 39.95, 64.93, 114.89 and 214.81 for 12.5, 25, 50 and 100 μM of PINO.

Cytotoxicity Assay

To assess the potential cytotoxic effects of PINO, MDA-MB-231, MCF7 and MCF10A cells were treated with concentrations of PINO ranging from 0.001 to 100 μM for 24 h. Surprisingly, PINO treatment was shown to promote a widespread cytotoxic effect at low concentrations in MCF7 cells and at all of the concentrations tested in MDA-MB-231 cells, although statistically significant changes were only observed from 0.001 to 1 μM (Figure 4). Importantly, the percentage of non-tumorigenic human mammary epithelial cells death following treatment with 0.001 μM PINO was much lower (10%) than in breast cancer cells (29% for MDA-MB-231 and 20% for MCF7 cells). In addition, a 10 μM PINO dose was shown to inhibit proliferation in MCF7 cells but did not induce significant cytotoxicity in MCF10A. Interestingly, a statistically significant cytotoxic effect was observed following 0.01 μM PINO treatment in both types of human breast tumour cells tested, but not in human mammary epithelial cells.

Cell proliferation

Proliferation of MDA-MB-231 (Figure 5.A), MCF7 (5.B) and MCF10A cells (5.C) was determined after treatment with PINO for 24 h followed by incubation with fresh medium. Measurements were performed at 24, 48, 72 and 96 h following treatment removal. At

0.001, 0.01 and 0.1 μM, cell survival was inhibited in MDA-MB-231 and MCF7 cells. Surprisingly, at 0.001 μM, proliferation was reduced in tumour cells, but not in mammary epithelial cells, in a statistically significant manner. Strong proliferation was observed in MCF10A cells treated with up to 100 μM PINO, whereas neither MDA-MB-231 nor MCF7 showed this effect.

Analysis of cell cycle and apoptosis

PINO treatment did not produce cell cycle alterations in the three cell lines studied, with the exception of the 100 μM concentration, which diminished the percentage of cells in the S phase in a statistically significant manner (Table 1). This percentage was 8.34 ± 0.94 vs. 17.01 ± 2.33 of the control for MDA-MB-231 (a decrease of 50.97 % respect to the control), 11.96 ± 0.68 vs. 16.72 ± 0.86 for MCF7 (decrease of 28.47%) and 9.92 ± 1.02 vs. 20.96 ± 1.29 for MCF10A (decrease of 52.67%). PINO also increased the percentage of cells in G0/G1 for MDA-MB-231 (73.39 ± 1.69 vs. 60.67 ± 4.8 of the control, i.e., increase of 20.97%) and MCF10A cells (76.1 ± 2.01 vs. 56.45 ± 0.2, that is, 34.81% of increase respect to the control) and decreased the percentage of cells in 47.24% in the G2/M phase in MCF10A cells (9.45 ± 2.1 vs. 17.91 ± 1.08 of the control). Unfortunately, 100 μM concentrations are not considered to be physiologically relevant. Representative cell cycle histograms of MDA-MB-231, MCF7 and MCF10A cells treated with PINO are shown in Additional file 1.

Statistically significant levels of apoptosis were induced in MCF10A cells treated with 100 μM PINO, with an increment of 445.86% respect to the control (7.26 ± 2.54 vs. 1.33 ± 0.42) (Table 2). An increase of 19.72% in apoptosis and 42.98% in cell death, albeit not statistically significant, also appeared in MDA-MB-231 cells treated at this concentration (18.46 ± 5.92 vs. 14.82 ± 4.76 and 3.42 ± 1.09 vs.

1.95 ± 0.6, respectively). No significant pro-apoptotic effects of PINO were reported in MCF7 cells. Additional file 2 represents the flow cytometry analysis of MDA-MB-231, MCF7 and MCF10A cells after treatment with PINO.

DCFH-DA

Reactive Oxygen Species (ROS) were measured using the DCFH-DA method under basal conditions (Figure 6.A) and after oxidative stress induced by H_2O_2 (6.B). In the basal state, 10 and 100 μM concentrations of PINO were shown to diminish ROS levels in a statistically significant way in MCF10A and MDA-MB-231 cells, whereas all concentrations of PINO decreased ROS levels in MCF10A cells (6.A). Under conditions of oxidative stress (6.B), the presence of ROS was increased in breast cancer cell lines, especially MCF7 cells, with statistically significant levels observed at 1, 10 and 100 μM in MCF7 cells and at 100 μM in MDA-MB-231 cells. Importantly, increased ROS production was not observed in PINO-treated human mammary epithelial cells (MCF10A).

Comet Assay

The percentage of DNA damage was determined by alkaline single-cell gel electrophoresis and expressed as Olive_TM. Data were expressed as the percentage relative to the basal (untreated) control, which was set as 100%. For MDA-MB-231 cells (Figure 7.A), DNA was injured by PINO under basal conditions at 0.1, 1 and 100 μM, but was protected after additional stress with H_2O_2. For MCF7 cells (7.B), treatment with PINO tended to increase DNA damage with respect to both the untreated control and H_2O_2-treated control; however, statistically significant changes were only observed at 100 μM. Finally, PINO treatment was shown to have more of a protective effect in

MCF10A cells (7.C) treated with H_2O_2 compared to the basal state. Indeed, a statistically significant reduction in DNA damage (93%) was observed at 1 μM. Figure 7.D shows representative comet assay images of MDA-MB-231, MCF7 and MCF10A cells under different conditions.

Discussion

As early as 1980s, it was suggested that lignans might prevent breast cancer and that this effect might be correlated with their phytoestrogenic activity. In addition, consumption of VOO, which contains significant amounts of lignans (e.g., PINO and 1-acetoxypinoresinol) as the major components of its phenolic fraction, has been correlated with a low occurrence of breast cancer [1]. In fact, in the phenolic fraction of VOOs there are several compounds with anti-breast cancer properties as oleouropein [33], hydroxytyrosol and tyrosol [6]. Certain compounds showed more effectiveness in ER- breast cancer cells than in ER+ breast cancer cells [33]. PINO and 1-acetoxypinoresinol were first detected in VOO by Owen et al. [8] and differ in their relative amounts according to the different olives varieties used to make the VOO [9]. For example, Brenes et al. [10] reported that Spanish olive oil contains a range of 20 to 45 mg/kg PINO. Despite the well-established preventative role of phytoestrogens against breast cancer, very little research has been done to elucidate whether PINO plays a chemopreventive role or exhibits antitumor activity in human breast cancer cells. Moreover, the oestrogen receptor status is a key factor to consider in breast cancer therapy. In fact, hormone therapy is only used in oestrogen receptor-positive breast cancer [17, 18]. Accordingly, we attempted to elucidate the effects of PINO on human mammary cells with different oestrogen and progesterone receptor expression, to determine

whether this compound may contribute, at least in part, to the reduced incidence of breast cancer associated with VOO consumption. For this purpose, we used the following human breast tumour cells: MDA-MB-231 cells (ER-, PR-) and MCF7 cells (ER+, PR+). Furthermore, non-tumorigenic human mammary epithelial cells were also used in the present study [MCF10A (ER-, PR-)].

Our results, summarized in Table 3, indicate that PINO showed cytotoxic, anti-proliferative and pro-oxidant activity in human breast tumour cells, independent of their oestrogen receptor expression levels. In addition, based on its effect in human mammary epithelial cells (Table 3), PINO may have chemopreventive activity, as induced antioxidant activity and prevented DNA from oxidative damage at a concentration of 1 μM. Interestingly, we found that PINO exerted differential activity on human breast tumour cells compared with mammary epithelial cells. Indeed, PINO treatment induced antioxidant activity in mammary epithelial cells, while it acted as a pro-oxidant molecule in human breast cancer cells after inducing oxidative stress.

The cytotoxic activity of PINO on human breast tumour cells is a debated issue. Previously, Chin et al. [25] described that PINO has a cytotoxic effect against MCF7 breast cancer cells (ED50 = 4.74 μM); however, in a later article [21], the same author found no cytotoxic effects. Surprisingly, the range of concentrations used in both studies was not specified. In addition, the cytotoxic effects of PINO in MDA-MB-231 cells have not been previously reported. In contrast, we tested a wide range of PINO concentrations and showed that there was cytotoxic activity at different concentrations in both human breast tumour cells tested. While PINO showed cytotoxic activity in both types of human breast tumour cells tested, the effect was more pronounced in negative oestrogen receptor tumour cells compared to oestrogen receptor-positive tumour cells (Figures 4 and 5). In addition, for the first time, we describe the effects of PINO on human

mammary epithelial cells. Our results suggest that PINO ranging between 0.001 and 0.1 μM, which could be considered as physiological doses, has a much greater cytotoxic effect on breast tumour cells compared to mammary epithelial cells, suggesting an anti-tumour effect of this compound with a minor damage to non-tumorigenic tissue.

Little research has been performed to understand the effects of PINO on human breast cancer cell proliferation. Sepporta et al. [27] found that PINO inhibited the growth of MDA-MB-231, but not of MCF7 cells; however, their study was limited to 100 μM, which is not considered to be a physiological concentration. In contrast, we tested a wide range of PINO concentrations, ranging from 0.001 to 100 μM, and showed that low concentrations of PINO elicited a significant antiproliferative effect on both human breast tumour cell lines tested. Future work is needed to clarify the mechanisms of inhibition of breast cancer cells growth only at low doses.

Oestrogen has been associated with the promotion and growth of breast cancer. In line with this result, most human breast cancers that are oestrogen-dependent undergo regression when deprived of the supporting hormone [17]. Our results, therefore, are very interesting because although PINO is a phytoestrogen with an oestrogen-like chemical structure, it produced a decrease in the proliferation of human breast tumour cells. Thus, PINO could have oestrogen antagonist activity, like tamoxifen, which inhibits breast cancer cells proliferation. However, in the experimental cell model we designed, we used cell culture media without oestrogen supplementation, suggesting that PINO is not likely to act as an oestrogen antagonist. Interestingly, a previous prospective study showed that high dietary intakes of plant lignans, such as PINO, were associated with reduced risks of ER+/PR+ postmenopausal breast cancer [20]. We do not believe that the anti-proliferative effects of PINO are mediated by interactions with ERα because this receptor is not expressed in MDA-

MB-231 breast tumour cells. Furthermore, the cell proliferation reduction was higher in MDA-MB-231 cells than in ERα+ MCF7 breast cancer cells. On the other hand, it is unlikely that the anti-proliferative effects of PINO could be due to the activation of ERβ because both breast cancer cells tested MDA-MB-231 and MCF7, express low levels of this receptor [34]. Additionally, it has been suggested that ERβ exerts anti-proliferative effects in breast cancer cells in the presence of ERα, but exerts proliferative effects in the absence of ERα [17]. If this were true, treatment with PINO would result in an increase of MDA-MB-231 breast cancer cell (ERβ low/ ERα negative) proliferation. Instead, we found an anti-proliferative effect, which was even greater than that observed in MCF7 breast tumour cells (ERβ low/ ERα positive). Based on these results, we hypothesize that the anti-proliferative effects of PINO in the breast cancer cells assayed are independent of both ERα and ERβ status.

Previously, it has been shown that persistent ROS induction in non-tumorigenic cells may lead to cancer initiation, progression and spreading via activation and maintenance of signalling pathways that regulate cellular proliferation, survival, angiogenesis and metastasis [35]. However, we have not found previously published results regarding the antioxidant capacity of PINO in mammary cells. Our results suggest that PINO may prevent cancer development, as it diminished ROS levels in MCF10A mammary epithelial cells.

On the other hand, it is known that cancer cells possess higher intracellular ROS levels than non-tumorigenic cells and that enhanced ROS levels may be exploited to promote cancer cell death [36]. In fact, many of the commonly used chemotherapies are based on increasing oxidative stress above a toxic threshold level to selectively kill cancer cells [36]. In line with this concept, PINO may be used as a potential effective adjuvant to cancer therapies, as it was found to promote ROS generation in breast cancer cells, while it tended to diminish ROS induction in mammary epithelial cells. MCF7 cells were shown to be

particularly sensitive to increased ROS levels after H_2O_2-induced oxidative stress, which could be related with the levels of DNA damage observed under basal conditions and after oxidative shock. Under basal conditions, PINO also caused DNA damage in MDA-MB-231 cells; however, in contrast, PINO treatment prevented DNA damage in non-tumorigenic mammary epithelial cells, suggesting that PINO treatment may protect DNA in a pro-tumorigenic environment, thereby inhibiting breast cancer initiation and progression. Surprisingly, ER negative cells showed reduced DNA damage in response to H_2O_2, whereas ER positive cells showed an increase in DNA damage.

Very few reports have studied the chemical antioxidant capacity of PINO, and the results have varied considerably. For example, Kuo et al. [22] obtained a significant DPPH free radical scavenging activity for PINO, but these results differ from the work done by Chin et al. [21] and Vuorela et al. [23], which demonstrated a much higher IC50. Our results suggest that PINO harbours a radical scavenging activity at concentrations of 10 μM or above for ABTS. This capacity was also shown using the DPPH method and is line with work published by Chin et al. [21]. In the ORAC assay, which is considered to be the most biologically relevant assay [24], PINO also showed antioxidant activity in a dose dependent manner.

Conclusions

Here, we showed that PINO possesses a chemical antioxidant capacity and may have a therapeutic potential to prevent breast cancer development via the reduction of intracellular oxidative stress and DNA damage in human mammary epithelial cells. Furthermore, we showed that PINO promotes an increase in the ROS levels of breast cancer cells after H_2O_2 treatment. In sum, this work suggests that PINO may act as adjuvant to pro-oxidative chemotherapies.

Finally, we showed that PINO has anti-tumour effects at low concentrations by promoting cytotoxic, anti-proliferative and pro-oxidant activities in breast cancer cells, independent of their oestrogen receptor status.

Abbreviations

ABTS: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DCFH-DA: dichlorofluorescin diacetate; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ER: oestrogen receptor; FBS: foetal bovine serum; HER2: human epidermal growth factor receptor 2; NEAA: non-essential amino acids; ORAC: oxygen radical absorbance capacity; PINO, (+)-pinoresinol; PR: progesterone receptor; ROS: reactive oxygen species; RSA: radical scavenging activity; VOO, virgin olive oil.

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Availability of data and materials

The relevant datasets supporting the conclusions of this article are included within the article. The whole data including all images obtained, histograms, analyses or raw data are available from the corresponding author on reasonable request.

Authors' contribution

Conception and design: J.J.G., A. L.-B., C. S.-Q.; Development of methodology: A. L.-B., C. S.-Q.; Sample processing: A. L.-B., C. S.-Q.; Analysis of data: A. L.-B., J.J.G., M. D.-R.; Writing of the manuscript: A. L.-B., J.J.G.; Revision of the manuscript: J.J.G., G.B., M. D.-R. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interest.

Consent to publish

Not applicable

Ethics approval and consent to participate

Not applicable

References

1. Toledo E, Salas-Salvado J, Donat-Vargas C, Buil-Cosiales P, Estruch R, Ros E, et al. Mediterranean Diet and Invasive Breast Cancer Risk Among Women at High Cardiovascular Risk in the PREDIMED Trial: A Randomized Clinical Trial. JAMA Intern Med 2015, 175(11):1752-1760.

2. Sanchez-Quesada C, Lopez-Biedma A, Warleta F, Campos M, Beltran G, Gaforio JJ. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of Olea europaea. J Agric Food Chem 2013, 61(50):12173-12182.

3. Warleta F, Campos M, Allouche Y, Sanchez-Quesada C, Ruiz-Mora J, Beltran G, et al. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Food Chem Toxicol 2010, 48(4):1092-1100.

4. Allouche Y, Warleta F, Campos M, Sanchez-Quesada C, Uceda M, Beltran G, et al. 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 2011, 59(1):121-130.

5. Sanchez-Quesada C, Lopez-Biedma A, Gaforio JJ. Maslinic Acid enhances signals for the recruitment of macrophages and their differentiation to m1 state. Evid Based Complement Alternat Med 2015, 2015:654721.

6. Warleta F, Quesada CS, Campos M, Allouche Y, Beltran G, Gaforio JJ. Hydroxytyrosol protects against oxidative DNA damage in human breast cells. Nutrients 2011, 3(10):839-857.

7. Martin-Pelaez S, Covas MI, Fito M, Kusar A, Pravst I. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol Nutr Food Res 2013, 57(5):760-771.

8. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Identification of lignans as major components in the phenolic fraction of olive oil. Clin Chem 2000, 46(7):976-988.

9. Allouche Y, Jimenez A, Gaforio JJ, Uceda M, Beltran G. How heating affects extra virgin olive oil quality indexes and chemical composition. J Agric Food Chem 2007, 55(23):9646-9654.

10. Brenes M, Garcia A, Garcia P, Rios JJ, Garrido A. Phenolic compounds in Spanish olive oils. J Agric Food Chem 1999, 47(9):3535-3540.

11. Kulik T, Busko M, Pszczolkowska A, Perkowski J, Okorski A. Plant lignans inhibit growth and trichothecene biosynthesis in Fusarium graminearum. Lett Appl Microbiol 2014, 59(1):99-107.

12. Yang CP, Huang GJ, Huang HC, Chen YC, Chang CI, Wang SY, et al. The Effect of the Aerial Part of Lindera akoensis on Lipopolysaccharides (LPS)-Induced Nitric Oxide Production in RAW264.7 Cells. Int J Mol Sci 2013, 14(5):9168-9181.

13. During A, Debouche C, Raas T, Larondelle Y. Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal Caco-2 cells. J Nutr 2012, 142(10):1798-1805.

14. Wikul A, Damsud T, Kataoka K, Phuwapraisirisan P. (+)-Pinoresinol is a putative hypoglycemic agent in defatted sesame (Sesamum indicum) seeds though inhibiting alpha-glucosidase. Bioorg Med Chem Lett 2012, 22(16):5215-5217.

15. Fini L, Hotchkiss E, Fogliano V, Graziani G, Romano M, De Vol EB, et al. Chemopreventive properties of pinoresinol-rich olive oil

involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. Carcinogenesis 2008, 29(1):139-146.

16. Hashim YZ, Rowland IR, McGlynn H, Servili M, Selvaggini R, Taticchi A, et al. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. Int J Cancer 2008, 122(3):495-500.

17. Haldosen LA, Zhao C, Dahlman-Wright K. Estrogen receptor beta in breast cancer. Mol Cell Endocrinol 2014, 382(1):665-672.

18. Ososki AL, Kennelly EJ. Phytoestrogens: a review of the present state of research. Phytother Res 2003, 17(8):845-869.

19. Leygue E, Murphy LC. A bi-faceted role of estrogen receptor beta in breast cancer. Endocr Relat Cancer 2013, 20(3):R127-39.

20. Touillaud MS, Thiebaut AC, Fournier A, Niravong M, Boutron-Ruault MC, Clavel-Chapelon F. Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. J Natl Cancer Inst 2007, 99(6):475-486.

21. Chin YW, Chai HB, Keller WJ, Kinghorn AD. Lignans and other constituents of the fruits of Euterpe oleracea (Acai) with antioxidant and cytoprotective activities. J Agric Food Chem 2008, 56(17):7759-7764.

22. Kuo PC, Lin MC, Chen GF, Yiu TJ, Tzen JT. Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. J Agric Food Chem 2011, 59(7):3214-3219.

23. Vuorela S, Kreander K, Karonen M, Nieminen R, Hamalainen M, Galkin A, et al. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. J Agric Food Chem 2005, 53(15):5922-5931.

24. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005, 53(10):4290-4302.

25. Chin YW, Jones WP, Rachman I, Riswan S, Kardono LB, Chai HB, et al. Cytotoxic lignans from the stems of Helicteres hirsuta collected in Indonesia. Phytother Res 2006, 20(1):62-65.

26. Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, et al. Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med 2008, 22(4):433-439.

27. Sepporta MV, Mazza T, Morozzi G, Fabiani R. Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. Nutr Cancer 2013, 65(8):1208-1218.

28. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999, 26(9-10):1231-1237.

29. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol. 1995, 28:25-30.

30. Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. J Agric Food Chem 2003, 51(11):3273-3279.

31. Sanchez-Quesada C, Lopez-Biedma A, Gaforio JJ. The differential localization of a methyl group confers a different anti-breast cancer activity to two triterpenes present in olives. Food Funct 2015, 6(1):249-256.

32. Lee DH, Lim BS, Lee YK, Yang HC. Effects of hydrogen peroxide (H2O2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. Cell Biol Toxicol 2006, 22(1):39-46.

33. Elamin MH, Daghestani MH, Omer SA, Elobeid MA, Virk P, Al-Olayan EM, et al. Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. Food Chem Toxicol 2013, 53:310-6.

34. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep 2000, 7(1):157-167.

35. Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. Biochem Pharmacol 2014, 92(1):90-101.

36. Nogueira V, Hay N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin Cancer Res 2013, 19(16):4309-4314.

Figures

Figure 1. Chemical structure of (+)-pinoresinol.

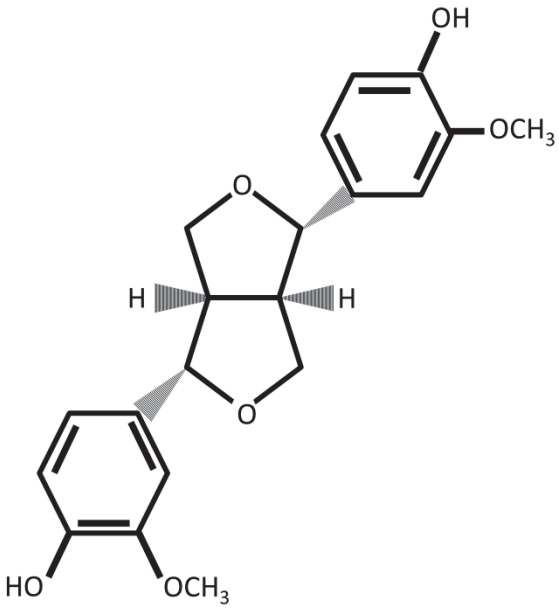


Figure 2. ABTS radical scavenging assay. $ABTS^{\bullet+}$ radical scavenging activity of (+)-pinoresinol (A) or Trolox™ (B) at concentrations between 0.01and 1000 μM and 50 and 800 μM, respectively. Data are expressed as the absorbance of $ABTS^{\bullet+}$, which was read at 734 nm for 2 h. Measurements were performed in triplicate in three independent experiments.

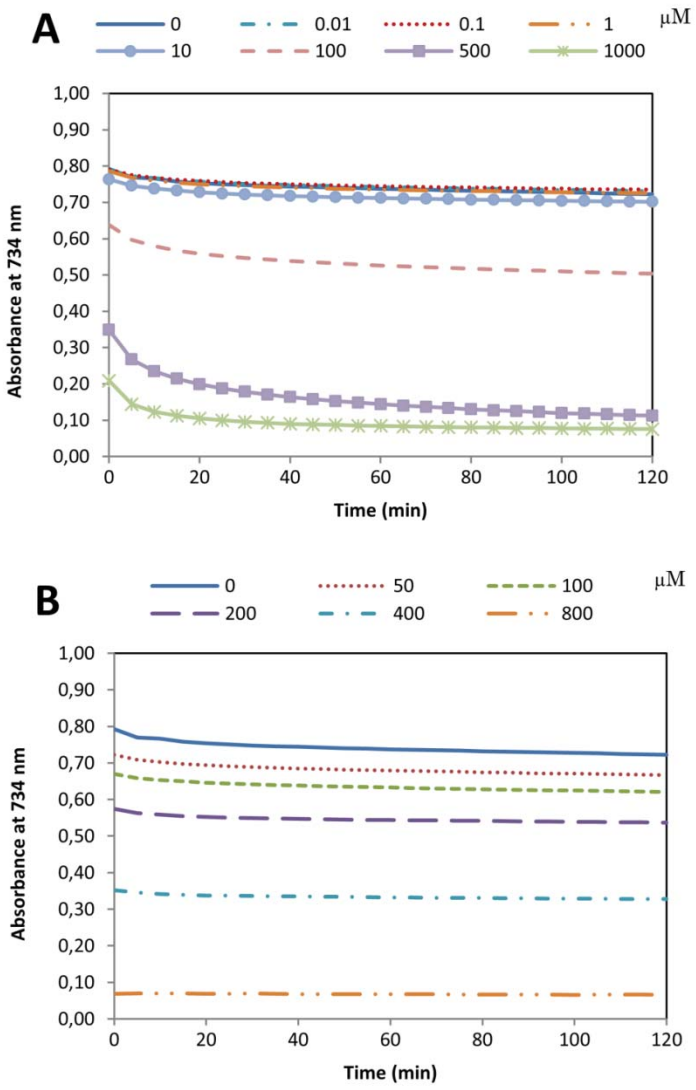


Figure 3. DPPH radical scavenging assay. Antioxidant activity of (+)-pinoresinol (A) against DPPH radicals at 0.03, 0.06, 0.13, 0.25, 0.5, 1 and 2 mole ratios (mol antioxidant/mol DPPH). α-tocopherol (B) was used as the antioxidant control at the same ratios. Data represents the absorbance of DPPH radicals read at 520 nm for 2 h. Each experiment was performed in triplicate and repeated three times.

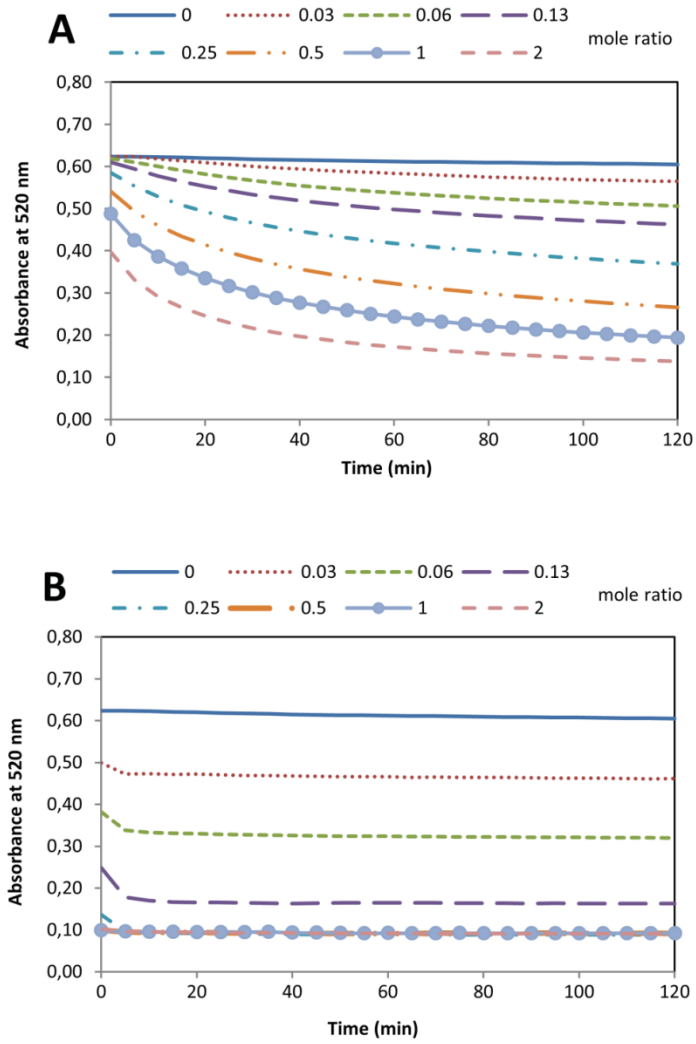


Figure 4. Cytotoxicity assay. Cell survival measured by CellTiter-Blue® after 24 h of (+)-pinoresinol treatment on MDA-MB-231, MCF7 and MCF10A cells. Data are represented as the treatment average (±SEM) with respect to the control, which was considered as 100%, for three independent assays carried out in triplicate. * MDA-MB-231, † MCF7 and Δ MCF10A indicate statistically significant differences at $p<0.05$.

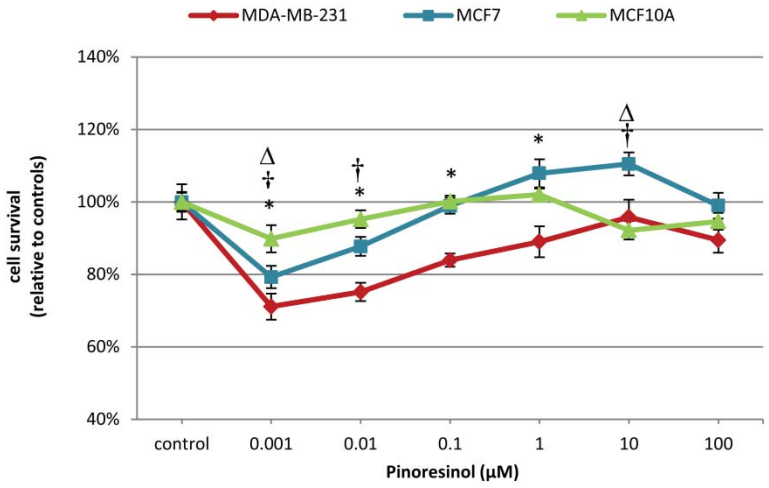
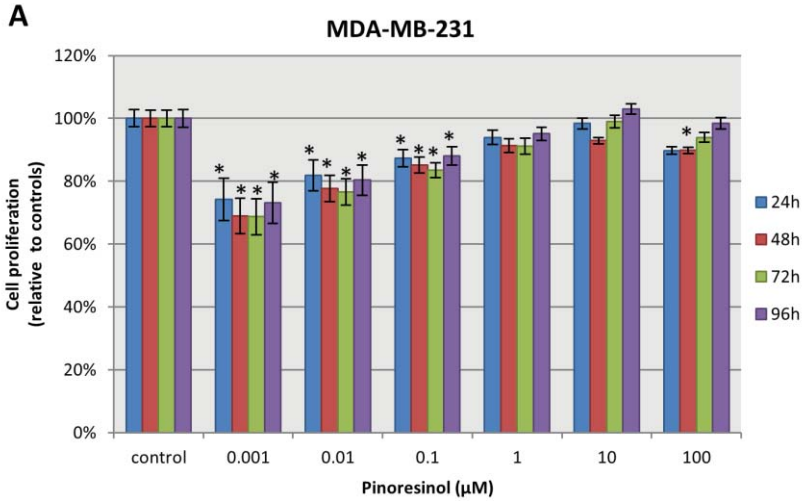
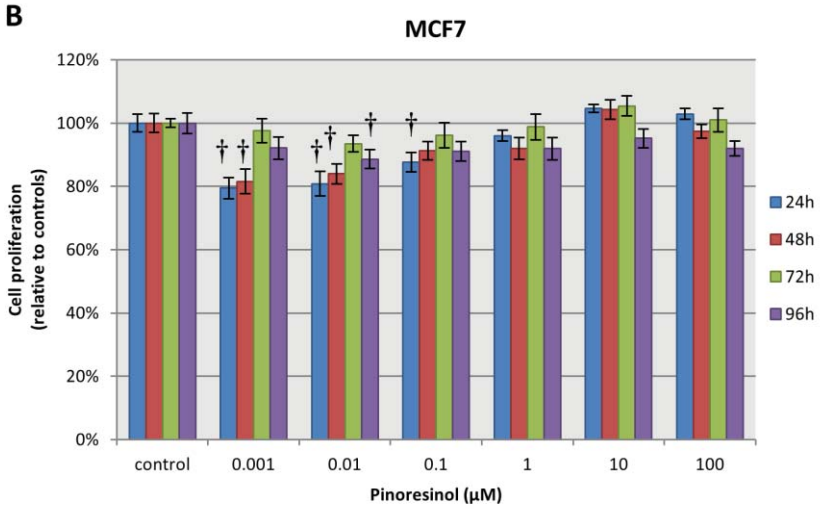


Figure 5. Cell proliferation. Cell proliferation was measured by CellTiter-Blue® after 24 h of (+)-pinoresinol treatment followed by proliferation periods of 24, 48, 72 and 96 h in MDA-MB-231 (A), MCF7 (B) and MCF10A (C) cells. Data are represented as the mean (±SEM) with respect to the controls, which were set as 100%, for three independent assays carried out in triplicate. *, † and Δ denote statistically significant differences relative to the control at $p<0.05$ for MDA-MB-231, MCF7 or MCF10A, respectively.




B
MCF7
120%
100%
80%
60%
40%
20%
0%
Cell proliferation (relative to controls)
24h
48h
72h
96h
control
0.001
0.01
0.1
1
10
100
Pinoresinol (μM)

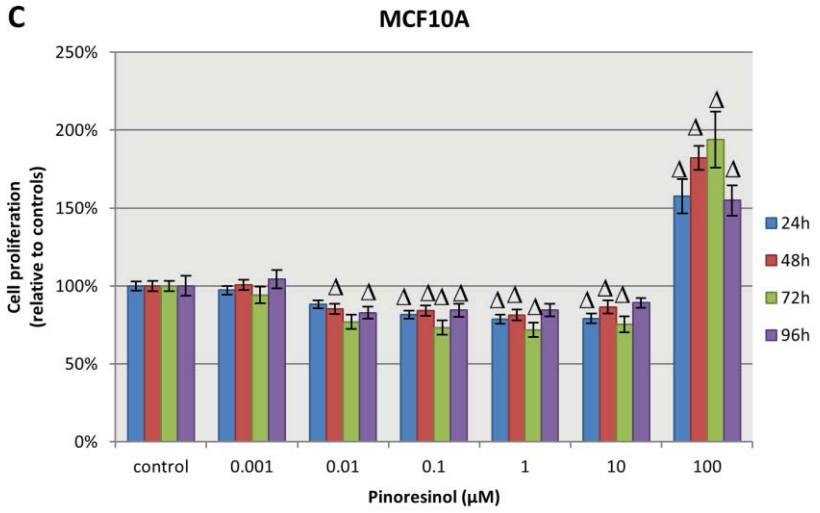

C
MCF10A
250%
200%
150%
100%
50%
0%
Cell proliferation (relative to controls)
24h
48h
72h
96h
control
0.001
0.01
0.1
1
10
100
Pinoresinol (μM)

Figure 6. DCFH-DA assay. Intracellular Reactive Oxygen Species (ROS) in breast cells treated with (+)-pinoresinol (in a range from 0.001 to 100 μM) for 24 h under basal conditions (A) and after H_2O_2-induced oxidative stress (B). Data are presented as the mean ± SEM of three independent experiments and *(for MDA-MB-231), † (for MCF7) and Δ (for MCF10A) represent statistically significant differences ($p<0.05$) with respect to the control, which was set as 100%.

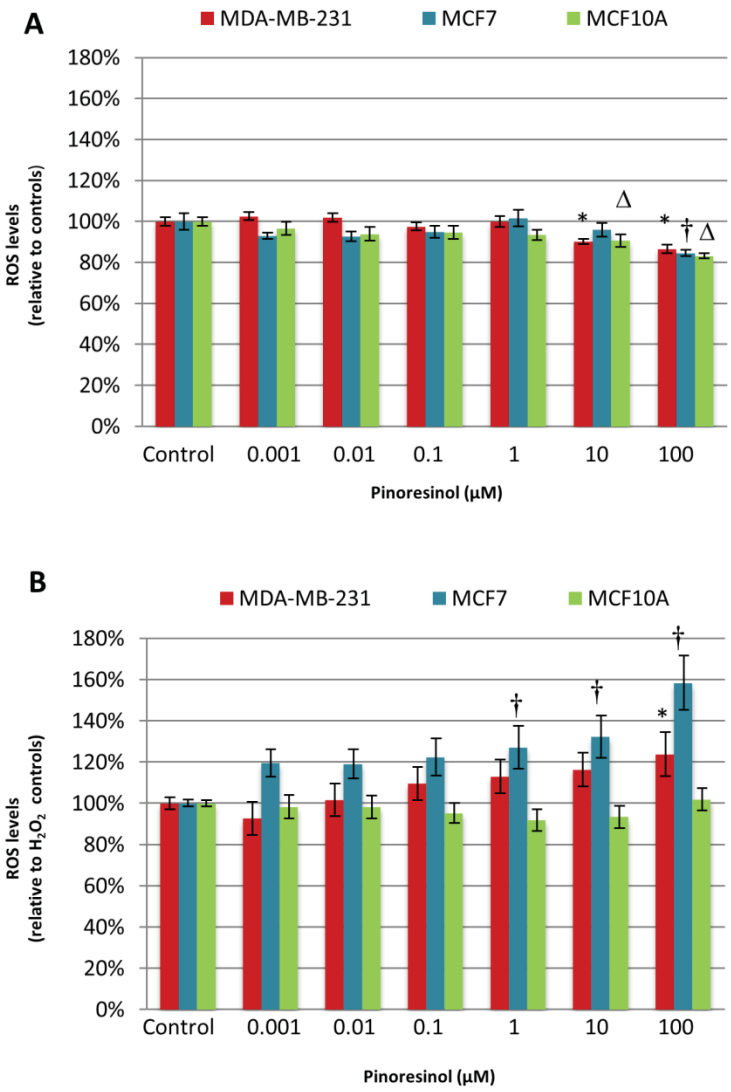
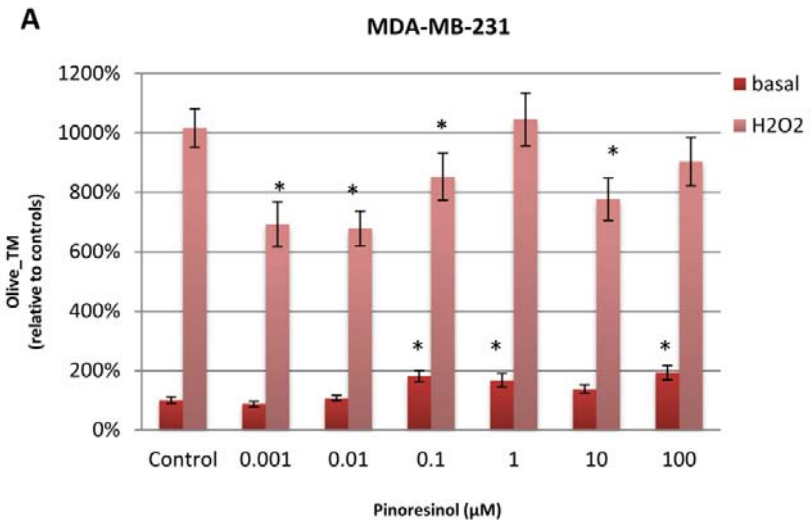
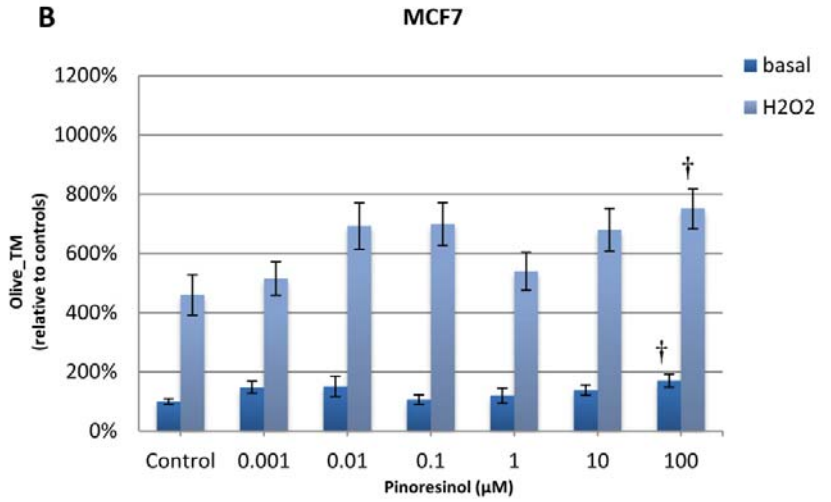
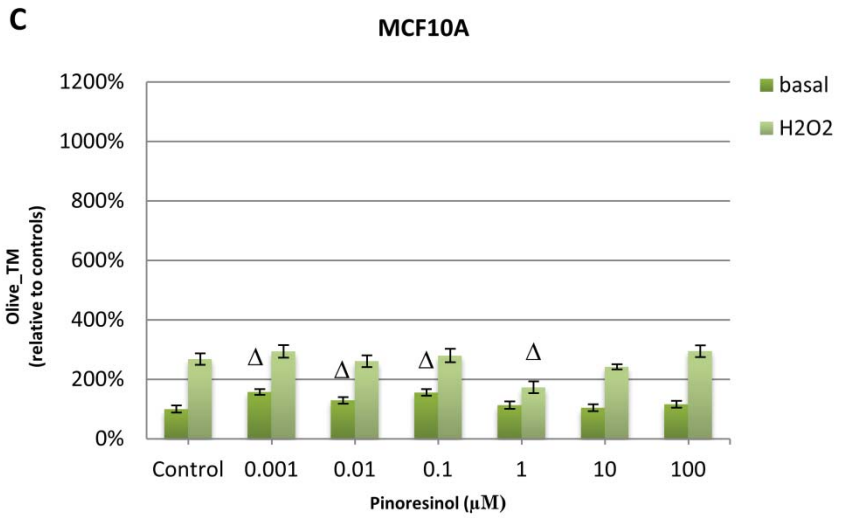


Figure 7. Comet assay. DNA oxidative damage in MDA-MB-231 (A), MCF7 (B) or MCF10A (C) after (+)-pinoresinol treatments expressed as Olive_TM (mean ± SEM). The comet assay was performed under basal and H_2O_2-induced injury conditions. Data are expressed as the percentage relative to the basal (untreated) control, which was set as 100%. Statistically significant differences (*, † or Δ) were established relative to basal or H_2O_2-treated control ($p<0.05$). (D) Representative comet assay images showing different treatments on MDA-MB-231, MCF7 and MCF10A cells.




B
MCF7
basal
H2O2
1200%
1000%
800%
600%
400%
200%
0%
Olive_TM
(relative to controls)
†
†
Control
0.001
0.01
0.1
1
10
100
Pinoresinol (μM)


C
MCF10A
basal
H2O2
1200%
1000%
800%
600%
400%
200%
0%
Olive_TM
(relative to controls)
Δ
Δ
Δ
Δ
Control
0.001
0.01
0.1
1
10
100
Pinoresinol (μM)

D

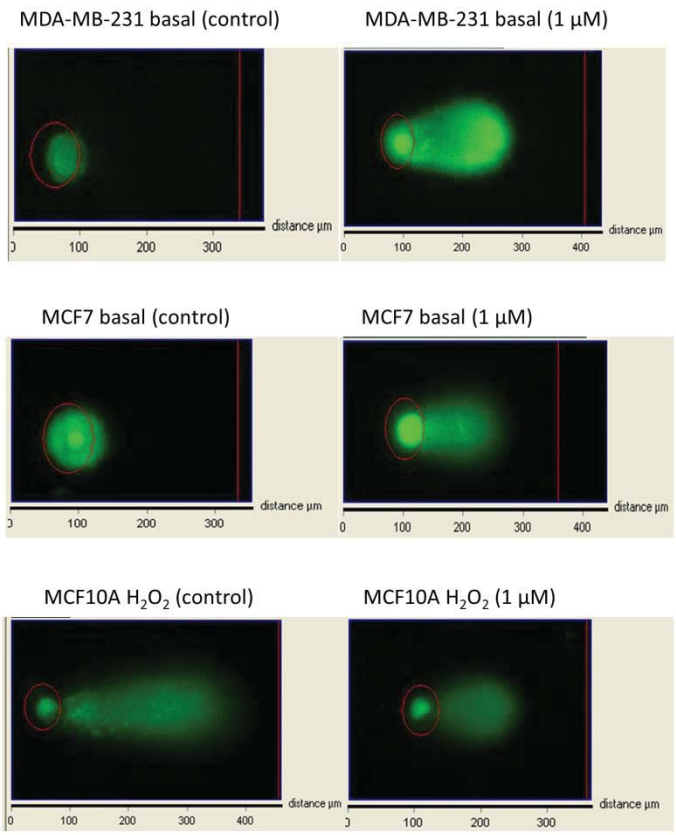

MDA-MB-231 basal (control)
MDA-MB-231 basal (1 µM)
distance µm
0
100
200
300
400
MCF7 basal (control)
MCF7 basal (1 µM)
distance µm
0
100
200
300
400
MCF10A H_2O_2 (control)
MCF10A H_2O_2 (1 µM)
distance µm
0
100
200
300
400

Table 1. Percentage of cell cycle phases was measured by flow cytometry after treatment with PINO. Values represent the percentage of the average ± SEM of three independent experiments. [a] indicates statistically significant differences with respect to control ($p<0.05$).

	MDA-MB-231				MCF7				MCF10A			
	SubG0/G1	G0/G1	S	G2/M	SubG0/G1	G0/G1	S	G2/M	SubG0/G1	G0/G1	S	G2/M
Control	1.72 ± 0.3	60.67 ± 4.8	17.01 ± 2.33	20.59 ± 2.46	0.69 ± 0.11	61.57 ± 0.19	16.72 ± 0.86	21.00 ± 0.74	1.23 ± 0.17	56.45 ± 0.2	20.96 ± 1.29	17.91 ± 1.08
0.001 µM	2.57 ± 0.7	60.87 ± 3.37	16.75 ± 1.68	20.23 ± 2.56	0.54 ± 0.03	62.71 ± 0.23	16.70 ± 0.43	20.60 ± 0.73	1.86 ± 0.7	55.32 ± 1.43	22.44 ± 0.34	16.77 ± 1.02
0.01 µM	1.80 ± 0.21	59.16 ± 4.53	17.55 ± 2.68	20.71 ± 2.01	0.62 ± 0.14	61.64 ± 1	16.72 ± 0.25	21.58 ± 0.93	1.67± 0.42	54.12 ± 2.3	21.79 ± 0.62	17.36 ± 1.47
0.1 µM	1.32 ± 0.24	59.33 ± 4.15	18.92 ± 1.68	20.35 ± 2.38	0.49 ± 0.02	60.71 ± 0.51	18.41 ± 0.5	20.61 ± 0.64	1.45 ± 0.22	54.84 ± 1.19	20.47 ± 0.53	19.09 ± 1.1
1 µM	1.62 ± 0.29	60.63 ± 4.93	18.56 ± 2.45	19.37 ± 2.48	0.49 ± 0.03	62.99 ± 1.28	16.99 ± 0.62	20.12 ± 1.06	1.85 ± 0.25	54.72 ± 0.65	21.23 ± 0.8	17.58 ± 1.16
10 µM	1.76 ± 0.3	64.85 ± 4.74	17.6 ± 2.38	15.52 ± 2.21	0.57 ± 0.03	61.73 ± 0.65	18.08 ± 0.41	19.62 ± 0.73	1.66 ± 0.12	53.93 ± 1.54	20.23 ± 1.1	19.6 ± 0.84
100 µM	2.29 ± 0.44	73.39 ± 1.69[a]	8.34 ± 0.94[a]	16.06 ± 1.8	0.81 ± 0.19	63.92 ± 1.13	11.96 ± 0.68[a]	23.38 ± 0.85	1.26 ± 0.23	76.1 ± 2.01[a]	9.92 ± 1.02[a]	9.45 ± 2.1[a]

	MDA-MB-231			MCF7			MCF10A		
	Live	Apoptotic	Death	Live	Apoptotic	Death	Live	Apoptotic	Death
Control	83.22 ±5.33	14.82 ± 4.76	1.95 ± 0.6	92,03 ± 2,69	6,97 ± 2,9	1,26 ± 0,61	98.14 ±0.47	1.33 ± 0.42	0.5 ± 0.08
0.001 µM	82.17 ± 4.76	15.56 ± 5.56	2.25 ± 0.94	92,38 ± 1,82	6,2 ± 1,83	1,07 ± 0,62	96.17 ± 0.76	2.73 ± 0.48	1.08 ± 0.29
0.01 µM	80.94 ± 8.15	16.24 ± 6.77	2.8 ± 1.57	93,14 ± 2,06	5,98 ± 1,69	0,85 ± 0,42	95.7 ± 1.44	3.45 ± 1.63	0.83 ± 0.37
0.1 µM	76.84 ± 9.32	20.15 ± 8.06	2.99 ± 1.34	92,47 ± 1,33	6,49 ± 1,08	1,03 ± 0,39	95.11 ± 2.6	3.99 ± 2.56	0.88 ± 0.16
1 µM	78.82 ± 7.41	18.05 ± 6.27	3.16 ± 1.29	93,32 ± 1,98	5,86 ± 1,61	0,8 ± 0,43	93.32 ± 1.51	5.85 ± 1.76	0.8 ± 0.25
10 µM	79.33 ± 7.24	17.75 ± 6.15	2.9 ± 1.19	93,89 ± 1,29	5,4 ± 1,08	0,69 ± 0,28	95.07 ± 1.1	4.17 ± 1.3	0.74 ± 0.2
100 µM	78.1 ± 6.84	18.46 ± 5.92	3.42 ± 1.09	91,65 ± 1,25	7,48 ± 1,14	0,85 ± 0,18	**91.82 ± 2.33[a]**	**7.26 ± 2.54[a]**	0.9 ± 0.23

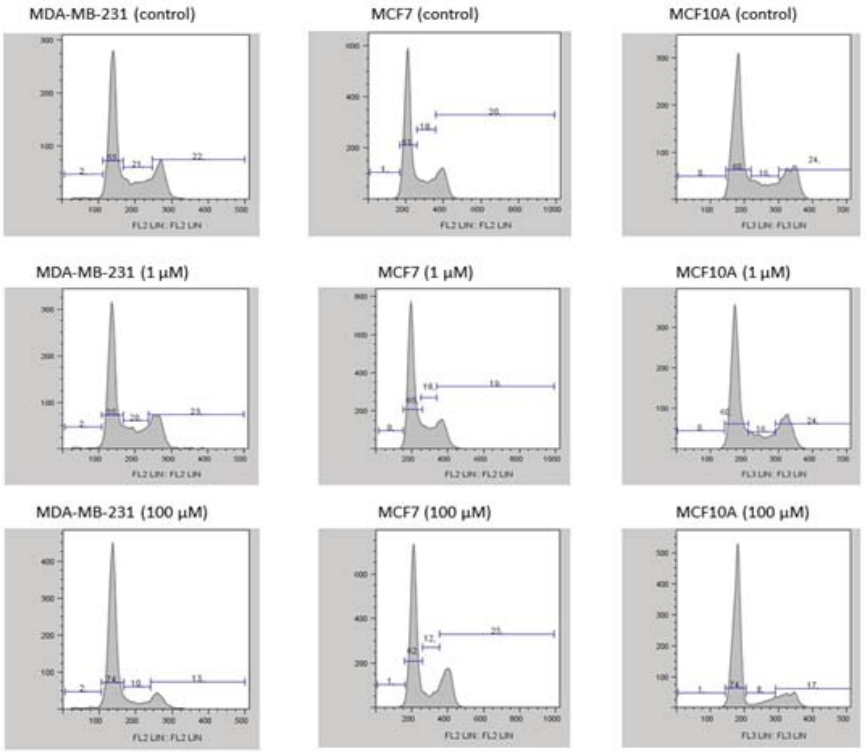
Table 2. Percentage of live, apoptotic and dead cells after 24h after exposure to PINO (0.001, 0.01, 0.1 1 10 and 100 µM). Values represent the average ± SEM of three independent experiments. [a] was considered statistically significant respect to the control ($p<0.05$).

A		Cytotoxicity	Proliferation	Oxidation (after H_2O_2 addition)	DNA damage (basal state)	DNA damage (after H_2O_2 addition)
MDA-MB-231 (ER-)	Concentrations (μM) with statistically significant differences	↑ 0.001 - 1	↓ 0.001 – 0.1	↑ 100	↑ 0.1, 1, 100	↓ 0.001 – 0.1, 10
MCF7 (ER+)	Concentrations (μM) with statistically significant differences	↑ 0.001 – 0.01	↓ 0.001 – 0.1	↑ 1 - 100	↑ 100	↑ 100

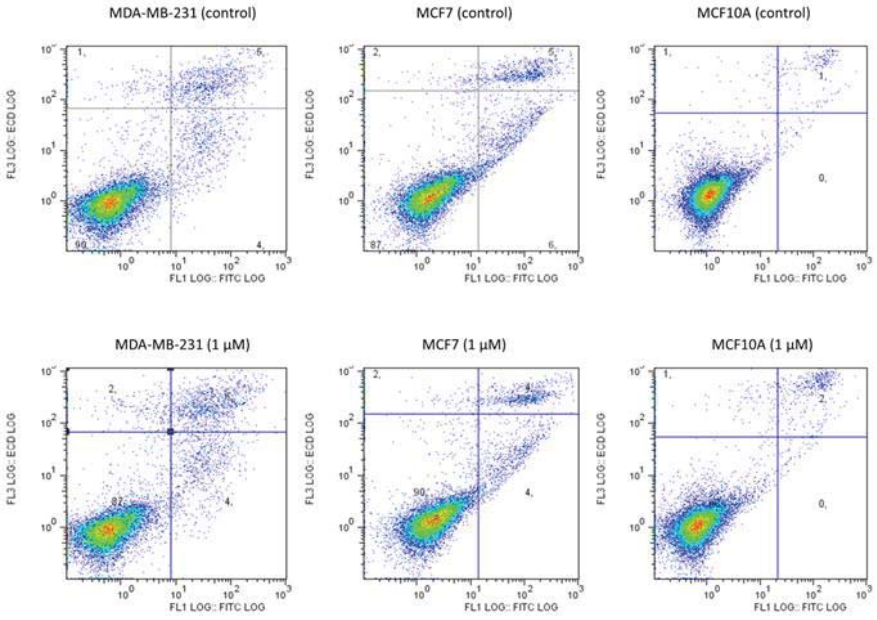
B		Cytotoxicity	Proliferation	Oxidation (basal state)	DNA damage (after H_2O_2 addition)
MCF10A (ER-)	Concentrations (μM) with statistically significant differences	↑ 0.001, 10	↓ 0.01 - 10	↓ 10, 100	↓ 1

Table 3. Summarized effects of PINO treatment on tumorigenic and non-tumorigenic mammary cells. a) Human breast tumour cells (MDA-MB-231 and MCF7) and b) non-tumorigenic human mammary epithelial cells (MCF10A).

Additional file 1. Representative flow cytometry analysis of the cell cycle of MDA-MB-231, MCF7 and MCF10A cells after treatment with (+)-pinoresinol.



Additional file 2. Representative images of apoptosis analysis by flow cytometry in MDA-MB-231, MCF7 and MCF10A cells.



3.2. Artículo 2

The biological activities of natural lignans from olives and virgin olive oils: A review.

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The biological activities of natural lignans from olives and virgin olive oils: A review.

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Abstract

Dietary guidelines recommend diets rich in plant foods, because they are associated with a lower incidence of chronic diseases, such as cardiovascular disease and certain cancers. The compounds that exhibit these health benefits are a matter of debate; however, scientific evidence assigns an important role to the action of so-called minor compounds. Lignans are polyphenols found in plants, and they are part of the phytoestrogen family, which is known for its health properties. The natural lignans (+)-pinoresinol and 1-acetoxypinoresinol are typically found in olives and, consequently, virgin olive oils (VOOs), which are genuine fruit juices. Although (+)-pinoresinol has been identified in other plants, 1-acetoxypinoresinol is specifically observed in olives. In this review, we collected information regarding these two main lignans found in VOOs, because a number of researchers believe that they may play a prominent role in the health effects attributed to virgin olive oils.

Keywords
(+)-pinoresinol, 1-acetoxypinoresinol, antioxidant, anti-inflammatory, metabolism, bioavailability.

1. Introduction

The incidence rates of certain chronic diseases, such as certain cancers and cardiovascular disease, are higher in Western countries, where people consume a diet rich in fat and animal protein, compared to countries that follow a Mediterranean diet rich in plant foods that contain phytoestrogens. Therefore, it has been postulated that phytoestrogens may be involved in conveying the beneficial effects attributed to the Mediterranean diet. In fact, phytoestrogens are abundant in the plasma and urine of subjects living in areas with a low cancer incidence rate (Adlercreutz, 2007).

The two main groups of phytoestrogens are isoflavonoids and lignans. It is widely assumed that the intake of lignan-rich foods is part of a healthy diet, and several reviews have conveyed information about lignans derived from different sources in the plant kingdom, as flaxseeds and sesame (Umezawa, 2003; Pan et al., 2009; Saleem, Kim, Ali, & Lee, 2005; Kajla, Sharma, & Sood, 2015; Dar & Arumugam, 2013). However, this is the first review that describes, specifically, the lignans typically found in olives and virgin olive oils (VOOs) and their implications on health and several diseases. These natural lignans found in olives and VOOs, which is the main source of fat in the Mediterranean diet, are (+)-pinoresinol and 1-acetoxypinoresinol. In fact, although (+)-pinoresinol has been identified in other plants, 1-acetoxypinoresinol is specifically found in olives. It is widely accepted that the consumption of lignans (Adlercreutz, 2007; Lin, Yngve, Lagergren, & Lu, 2012; Peterson et al., 2010) and VOOs (Toledo et al., 2015), provide health benefits, and several studies have highlighted beneficial effects of (+)-pinoresinol and 1-acetoxypinoresinol

characteristics of VOOs (Owen et al., 2000a; Menendez et al., 2008a). Consequently, both (+)-pinoresinol and 1-acetoxypinoresinol represent a high interest based on their biological and pharmacological properties. Unfortunately, the information known about these compounds is largely ambiguous and diverse, making more work for researchers who want to study and gather information on these compounds. Thus, this review summarizes the current published information related to the bioactivity and potential health benefits associated with (+)-pinoresinol and 1-acetoxypinoresinol, as well as their chemical characteristics, distribution and metabolism in humans. In addition, this study highlights the importance of further studying these compounds, due to their pharmacological potential.

2. Classification, Chemical Structure and Metabolic Pathway

Polyphenols are secondary metabolites in plants that are involved in the defence against ultraviolet radiation and aggressive pathogens (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). This group includes the lignans pinoresinol and 1-acetoxypinoresinol, the subjects of this review.

Specifically, pinoresinol ($C_{20}H_{22}O_6$) and 1-acetoxypinoresinol ($C_{22}H_{24}O_8$) are phenolic compounds that form a dimer, which means they possess two phenol groups in their chemical structure (**Figure 1**). Each phenol group consists of an aromatic ring (phenyl or benzene group) bound to a hydroxyl group (OH). Both, the phenol group and the benzene ring are associated with several health benefits in humans including antioxidant and/or anti-inflammatory effects (During, Debouche, Raas, & Larondelle, 2012; Sok, Cui, & Kim, 2009; Yang et al., 2013).

The molecular weights of pinoresinol and 1-acetoxypinoresinol are 358.38 and 416.42 g/mol, respectively. Their structures are very similar, except for the appearance of a -$COOCH_3$ group in 1-acetoxypinoresinol that is not present in pinoresinol.
The biosynthesis of pinoresinol has been investigated in *Forsythia* spp (Kim et al., 2009; Umezawa, 2003). This compound is synthesized by the stereospecific coupling of two units of coniferyl alcohol in the presence of a dirigent protein (DIR). It is then metabolized to lariciresinol and secoisolariciresinol via pinoresinol-lariciresinol reductase (PLR) or to piperitol and sesamin via piperitol/sesamin synthase (PSS). No references have reported the biosynthesis of 1-acetoxypinoresinol.

3. **Distribution**

Lignans are distributed widely in the plant kingdom, with flaxseeds being a major source (Thompson, Chen, Li, Strasser-Weippl, & Goss, 2005). Despite this fact, 1-acetoxypinoresinol has not been identified in flaxseeds and pinoresinol is present at very low concentrations in comparison to other lignans. It has been reported that the contribution of flaxseeds to the mean intake of pinoresinol is only 0.3% (Milder et al., 2005). 1-acetoxypinoresinol has been found only in the bark of the olive tree (*Olea europaea*) (Tsukamoto, Hisada, & Nishibe, 1984), while pinoresinol has been found in several species including *Forsythia* spp., *Linum flavum, Brassica* spp., *Daphne* spp., *Larix leptolepis*, *Sesamun indicum, Pinus* spp., *Picea jezoensis, Tsuga heterophylla, Araucaria angustifolia, Fraxinus mandshurica, Wikstroemia* spp., *Osmanthus fragans*, *Trichosanthes kirilowii*, *Sophora tonkinensis*, *Piper wallichii*, the stems of *Viburnum erosum*, the seeds of *Melia azedarach* and the roots of *Rosa multiflora*, among others (Carpinella, Giorda, Ferrayoli, & Palacios, 2003; Harborne, Baxter, & Moss, 1999; In et al., 2015; Lee et al., 2015; Liu et al., 2015; Minh et al., 2015; Owen et al., 2000b; Shi et al., 2015; Sok et al., 2009; Umezawa, 2003; Yeo, Chin, Park, & Kim, 2004).

In the year 2000, two independent studies (Brenes et al., 2000; Owen et al., 2000a) have identified, for the first time, pinoresinol and 1-acetoxypinoresinol in VOO; it was the enantiomer form of (+)-pinoresinol that was identified. This finding has attracted special interest, because the consumption of VOO is associated with beneficial properties that could result from the presence of these lignans as predominant compounds in its phenolic fraction. In fact, Owen et al. (2000a, 2000b) noted that these compounds are not present (or virtually absent) in seed oils and refined oils, but they are major components of the phenolic fraction of virgin olive oils, with concentrations up to 100 mg/kg. These researchers also found considerable variation in the concentrations of these compounds within different oils, but the average total lignan concentration was 41.53 ± 3.93 mg/kg. Similarly, Brenes, Garcia, Dobarganes, Velasco & Romero (2002a) found differences among 10 extra virgin olive oils, with concentrations varying from 11.7 to 41.2 mg/kg for (+)-pinoresinol and 2.7 to 66.9 mg/kg for 1-acetoxypinoresinol.

Since then, several studies have addressed the quantification of total phenolic or lignan content in VOO, as well as the concentrations of individual phenolic compounds. However, it is difficult to provide concrete data due to the high number of variables that can affect the concentrations of these compounds. These variables include olive variety, cultivation area, climate, harvesting, degree of ripening, stoning, production techniques, transport, storage and experimental procedures for isolation and analysis.

Time, temperature and oxygen concentration during malaxation (beating of the paste) also affect the phenolic concentrations (Servili et al., 2007), although some researchers did not find significant changes in the concentration of (+)-pinoresinol or 1-acetoxypinoresinol under different conditions of malaxation (García et al., 2001). In addition, Gambacorta et al. (2010) reported that the degree of maturation did

not have a significant influence on the concentration of 1-acetoxypinoresinol, as Bonoli, Bendini, Cerretani, Lercker and Toschi (2004) noted for both compounds. However, these researchers reported differences related to the technology used to obtain the VOO, because oils from stoned olives showed higher concentrations of 1-acetoxypinoresinol, which was also reported by Servili et al. (2007); the latter case, the variations were not significantly different. Gambacorta et al. (2010) also noted that 1-acetoxypinoresinol concentrations were influenced by storage time, resulting in a slight increase after 6 months of storage and a significant decrease after 12 months of storage.

A previous study (Kalua, Bedgood, Bishop, & Prenzler, 2008) reported that levels of pinoresinol diminished gradually during 4 weeks of storage, while 1-acetoxypinoresinol levels were enhanced by weeks 1 and 3. In contrast, Brenes, Garcia, Garcia and Garrido (2001) found that concentrations of pinoresinol and 1-acetoxypinoresinol did not change during a one-year storage period under commercial conditions (darkness and lack of oxygen). These researchers also observed minor losses of both compounds when VOO was heated, which is an important aspect considering that VOO is not only consumed raw but is also heated during frying, boiling or microwave heating (Brenes et al., 2002a).

In short, pinoresinol and 1-acetoxypinoresinol appear more stable than other phenolic compounds under different conditions of malaxation, olive stoning, maturity or storage time (Bonoli et al., 2004; Brenes et al., 2001; García et al., 2001; Servili et al., 2007). However, given the high number of variables involved in obtaining the final oil product, the concentrations of these lignans in VOOs differ widely.
Despite variations in lignan concentrations, lignans exist in certain proportions in different oil varieties. For example, 1-acetoxypinoresinol is very abundant in Arbequina, Empeltre and Hojiblanca oils but is present in very low concentrations in Picual oils.

Therefore, this compound has been proposed as a potential indicator of authenticity for Picual oils (Brenes, García, Rios, García, & Garrido, 2002b; Gomez Caravaca, Carrasco Pancorbo, Canabate Diaz, Segura Carretero, & Fernandez Gutierrez, 2005). For guidance, **Table 1** gives the concentrations found in different oil varieties.

The location of these compounds inside the olive tree remains unclear. Some researchers, such as Owen et al. (2000b), found that these lignans are not present in the pericarp of the olive drupes or in the leaves and twigs that are present in the mulch prepared for olive pressing. However, (+)-pinoresinol was found to be a major component of the phenolic fraction of olive piths. Furthermore, Bonoli et al. (2004) found pinoresinol and a small amount of 1-acetoxypinoresinol in the bark of olive fruit trees from Italian cultivars, but none of the compounds was detected in the extracts of seeds and pulp. Similar results were obtained by Oliveras-López et al. (2008) who found pinoresinol in the olive stones of four olive varieties. They did not find pinoresinol or 1-acetoxypinoresinol in the pulp, although a small amount of 1-acetoxypinoresinol was found in the Taggiasca variety. Servili et al. (2007) detected lignans in both the pulp and the stone but not in the seeds of olive fruits. 1-acetoxypinoresinol was the more prevalent lignan in the pulp, and pinoresinol was the more prevalent lignan in the stone. By contrast, Kalua et al. (2008) detected these lignans in olive oil but not in the olive fruits of the Frantoio variety.

It is worth noting that although these lignans are sometimes present in VOOs, they are not found in the fruits from which the oils originate. One plausible explanation could be that the techniques used for extraction in these studies failed to detect the compounds. For instance, Oliveras López et al. (2008) tried several methods to extract pinoresinol and 1-acetoxypinoresinol from stones and pulp of Italian and Spanish olive varieties known to produce VOOs rich in both lignans. Surprisingly, only one of the extraction methods was able to

yield detectable levels of pinoresinol in the stones of the selected varieties. Another possible explanation could be that lignans, initially absent in olive fruits, are generated by the activity of β-glucosidase and the acidic conditions that arise when the olives are pasted during olive oil production (Soler et al., 2010). Finally, it is possible (though less likely) that lignans present in other parts of the olive tree that are included in the olive oil production process, such as stones, twigs and leaves, become incorporated in the final olive oil product.

4. **Bioactivity**

Lignans have been associated with several health properties such as protection against LDL oxidation and inhibition of cancerous cell growth in skin, breast, prostate, colon and lung tissues (Hirano et al., 1990; Kardono, Tsauri, Padmawinata, Pezzuto, & Kinghorn, 1990). Because lignans have chemical structures similar to oestrogen, they could act as hormonal modulators in breast cancers through oestrogenic or antiestrogenic activities. In fact, clinical trials have reported a reduction in the risk of postmenopausal breast cancer, specifically the ER^+/PR^+ subtype, in women with a high intake of plant lignans (Touillaud et al., 2007) and a reduction in breast tumour growth in patients who consume flaxseed daily (Thompson et al., 2005).

However, due to the broad range of compounds included in the lignan group, it would be advantageous to study the properties and biological activities of each one individually, because small variations in chemical structure may cause different or even opposite effects.

4.1 Free radical scavenging activity

Experimental data on 1-acetoxypinoresinol is fairly limited. This compound is not commercially available and difficulties in its isolation

and synthesis have limited the study of its effects. Regarding its potential as a free radical scavenger, this compound elicited stronger responses than the standards Trolox and dimethyl sulphoxide in reactive oxygen species (ROS) attacks on salicylic acid in hypoxanthine/xanthine oxidase assays, where the inhibitory concentrations (IC_{50}) were 0.91, 12.24 and 2.30 mM for 1-acetoxypinoresinol, Trolox and dimethyl sulphoxide, respectively (Owen et al., 2000a). This same study also reported that 1-acetoxypinoresinol had a higher radical scavenging activity than the catechol derivatives caffeic acid, hydroxytyrosol and oleuropein.

Research using the DPPH method showed that both lignans were antioxidants, but (+)-pinoresinol had a greater effect than 1-acetoxypinoresinol (Carrasco-Pancorbo et al., 2005). However, these authors noted that both compounds had a pro-oxidant effect when used in a lipid model system (OSI), this effect being much higher for (+)-pinoresinol. As mentioned above, the chemical structure of this compound could justify the results of this study, because the –$COOCH_3$ group is not present in (+)-pinoresinol (Figure 1). This group is not an electron donor, which may explain the lower antioxidant capacity of 1-acetoxypinoresinol in DPPH assays. Moreover, the pro-oxidant effect observed with the OSI method could be due to the oxygen atoms in each central ring contained in both molecules, which could cause an opening of the ring under the thermal conditions used in the assay. However, the –$COOCH_3$ group of 1-acetoxypinoresinol could hinder the opening of the ring, producing a weaker effect (Carrasco-Pancorbo et al., 2005).

Once again, the method of measurement seems to be the cause of conflicting results, but there are also differences among results derived from the same procedure. For example, with the DPPH assay, Kang and Wang (2010) observed high levels of radical scavenging activity for (+)-pinoresinol using similar concentrations of compounds to Kuo, Lin, Chen, Yiu and Tzen (2011)(IC_{50} values were 24.16 μM by

Kang and Wang (2010), and 34.5 μM by Kuo et al. (2011)). However, these values were much lower than those obtained by Chin, Chai, Keller and Kinghorn (2008) (IC_{50} = 96.82 μM), Yi et al. (2011) (IC_{50} = 558 μM) and Vuorela et al. (2005) (percent of DPPH inhibition ± SD = 18.8 ± 1.3 for 1395 μM). Moreover, Li, Zhai, Tang and Duan (2010) found no antioxidant activity for (+)-pinoresinol using this method. For ABTS testing, a recent study detected a noticeable radical scavenging activity (Koch, Buchter, Havermann, & Watjen, 2015). Kang and Wang (2010) and Yi et al. (2011) also observed significant antioxidant activity, but IC_{50} values varied considerably (8.6 μM (Kang & Wang, 2010) and 153.46 μM (Yi et al., 2011)).

Data related to the radical scavenging activities of (+)-pinoresinol and 1-acetoxypinoresinol are shown in **Table 2**.

4.2 Biological activity in vitro

In vitro studies showed that 1-acetoxypinoresinol completely reduced the fatty acid synthase (FASN) levels in HER2 gene-amplified SKBR3 breast cancer cells, which naturally overexpress this protein, and in MCF7 breast cancer cells engineered to overexpress the HER2 tyrosine kinase receptor (Menendez et al., 2008a). (+)-pinoresinol also diminished these levels, although to a lesser extent (30-35% reduction for SKBR3 cells and 50% for MCF7 cells). FASN protein levels are frequently altered in cancer cells, and this protein has been implicated in the activity and/or expression of key cancer-related oncogenes such as the Type I receptor tyrosine kinase HER2, whose overexpression enhances breast cancer cell proliferation, survival, chemoresistance and metastasis. Therefore, 1-acetoxypinoresinol and (+)-pinoresinol could be useful in the chemoprevention and/or treatment of breast carcinomas with FASN overexpression that result from oncogenic signalling driven by HER2 (Menendez et al., 2008b). The same authors reported significant cytotoxicity in SKBR3 cells, as well as reduced cell proliferation, apoptosis and inhibition of HER2 protein kinase

activity, when VOO phenolic fractions were applied (Menendez et al., 2008a). The phenolic fraction, containing mainly 1-acetoxypinoresinol, was more effective than the fraction that contained mainly (+)-pinoresinol. Moreover, in MCF7/HER2 cells, the first phenolic fraction increased apoptosis greater than 4-fold and reduced the proliferation rate by 63% and HER2 expression by 83% (Menendez et al., 2008a). However, it is not known whether these effects were due to the presence of 1-acetoxypinoresinol or (+)-pinoresinol, because, the fractions contained small quantities of other polyphenolic compounds that could be contributing to the above mentioned activities.

Menendez et al. (2009) studied the effects of these lignans individually on breast epithelial cells (MCF10A) engineered to overexpress the wild-type form of human HER2. Remarkably, the compounds were able to down-regulate HER2 protein expression (≈30% reduction for (+)-pinoresinol and >70% for 1-acetoxypinoresinol), but further HER2 tyrosine kinase activity was decreased after the treatments, up to 70% in the case of 1-acetoxypinoresinol. The IC_{50} values required to decrease HER2 Y1248 phosphorylation were 64 μM of (+)-pinoresinol and 30 μM of 1-acetoxypinoresinol. Furthermore, the morphology of treated MCF10A/HER2 cells underwent striking changes including diminished growth density, expanded volume and the appearance of extrusions. Exposure to 1-acetoxypinoresinol inhibited the growth of cells and resulted in cell morphologies compatible with apoptosis. The authors suggested that the inhibitory effects were due to the presence of two or more phenol rings, because the single phenols and phenolic acids studied did not efficiently block HER2 tyrosine kinase activity (Menendez et al., 2009).

To date, few references have addressed the *in vitro* or *in vivo* activities of 1-acetoxypinoresinol. Studies have been carried out for pinoresinol, most of which have reported activities including antifungal, anti-inflammatory, antioxidant or hypoglycaemic activities (During et al.,

2012; Jung et al., 2010; Kulik, Busko, Pszcsolkowska, Perkowski, & Okorski, 2014; Wikul, Damsud, Kataoka, & Phuwapraisirisan, 2012).
It is difficult to reach conclusions with respect to the possible cytotoxic effect of pinoresinol on cancer cells, because results vary broadly depending on the cell line used. Chin et al. (2006) observed that pinoresinol was cytotoxic in MCF7 (human breast cancer), LNCaP (hormone-dependent human prostate cancer) and Lu1 (human lung cancer) cells. In contrast, in a later article (Chin et al., 2008), the same authors found no cytotoxic effects in MCF7 cells, even observed cell cytoprotection when stressed the cells by H_2O_2. Previously, pinoresinol exhibited cytotoxicity in KB (HeLa derivative) cells with an IC_{50} = 2.2 μg/mL (Chiung et al., 1994), but no activity was observed in HL60 human promyelocytic leukaemia cells (Mimaki, Kuroda, Asano, & Sashida, 1999).

Fini et al. (2008) observed that pinoresinol inhibited cell viability and arrested the cell cycle in G2/M phase only in p53-proficient cells when tested in colon cancer cell lines with different expression levels of p53 gene. A synergistic effect together with other polyphenols present in the phenolic fraction of VOOs was suggested by these authors, because lower concentrations of pinoresinol were necessary to achieve cytotoxicity when using pinoresinol-rich extracts on human colon cancer cells. The use of these extracts decreased cell viability and induced apoptosis and G_2/M cell cycle arrest, presumably through the upregulation of ATM/p53 pathway. However, after using 100 μM of (+)-pinoresinol by itself, Sepporta, Mazza, Morozzi and Fabiani (2013) noted that the antiproliferative activity was not always linked to the presence of functional p53. In human breast cancer cells, these researchers obtained this same effect in a cell line with a p53 mutation (MDA-MB-231) but not in p53-proficient cells (MCF7). In fact, they tested a panel of p53 proficient and deficient cell lines and, contrary to Mimaki et al. (1999), p53 deficient HL60 cells were the most sensitive to growth inhibition, with an IC_{50} = 8 μM.

The $p21^{WAF1/Cip1}$ protein, which is a modulator of biological functions associated with proliferation, apoptosis and differentiation, seems to play a role in the growth of cells treated with (+)-pinoresinol, which strongly induced its expression at both the protein and mRNA levels, resulting in antiproliferative effects (Sepporta et al., 2013). Cytotoxicity has also been reported in lung adenocarcinoma (A549), ovarian adenocarcinoma (OK-OV-3), skin melanoma (MEL-2) and colon carcinoma (HCT15) cells with IC_{50} values ranging from 26-34 μg/mL (Kwak, Kang, Roh, Choi, & Zee, 2009).

Despite the relevance of invasion to cancer progression and metastasis, there is only one study that covers the possible anti-invasive effects of pinoresinol. This study was carried out on human colon adenocarcinoma cells (HT115), in which pinoresinol showed significant anti-invasive effects when used at a range of concentrations from 1.56 to 25 μM, with a reduction of up to 65% when used at its lowest concentration (Hashim et al., 2008).

As an antifungal, pinoresinol was able to inhibit the growth of the pathogenic fungi *Fusarium verticilloides* (Carpinella et al., 2003) and *Fusarium graminearum*, as well as decreased trichothecene biosynthesis (Kulik et al., 2014). Hwang, Lee, Liu, Woo and Lee (2010) also described pinoresinol's effectiveness as an antifungal, with minimum inhibitory concentrations (MIC) of 12.5 μg/mL for the human fungi *Candida albicans* and 25 μg/mL for both *Trichosporon beigelii* and *Malassezia furfur*. These values represent a less potent antifungal activity than the positive control Amphotericin B, which is a fungicidal agent used to treat several life-threatening infections in humans. Likewise, pinoresinol did not exhibit haemolytic activity against human erythrocytes, which is one of the side effects associated with Amphotericin B (Hwang et al., 2010).

However, (+)-pinoresinol did inhibit $CuSO_4$-induced peroxidation of low-density lipoprotein (LDL) in a concentration-dependent manner

from 0.1 μM to 10 μM (Kang, Naito, Sakai, Uchida, & Osawa, 2000). The oxidation of LDL plays an important role in the formation of atherosclerotic plaques, and thus lignans could be used to reduce the appearance of the first signs of atherosclerosis in cardiovascular disease (Rafieian-Kopaei, Setorki, Doudi, Baradaran, & Nasri, 2014).
(+)-pinoresinol was also effective as an antioxidant for liposomes, as well as an anti-inflammatory agent through its inhibition of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as observed in activated J774 murine macrophages (Vuorela et al., 2005). Jung et al. (2010) also reported pinoresinol anti-inflammatory activity in the primary microglia of rats, along with the inhibition of other proinflammatory mediators such as COX-2, TNFα, IL1β, IL-6 and NF-κB. During et al. (2012) observed a decrease in IL-6, PGE_2 and NF-κB levels after treatment in human colon adenocarcinoma cells (Caco-2). These authors associated the anti-inflammatory properties of pinoresinol with its furofuran structure, given that other lignans without this structure did not produce these effects. Several recent studies performed in LPS-activated RAW264.7 macrophages confirmed that NO production could be inhibited by pinoresinol, albeit with varying IC_{50} values (In et al., 2015; Lee et al., 2015; Liu et al., 2015; Yang et al., 2013). Therefore, (+)-pinoresinol could potentially be used to treat inflammatory diseases, specifically those associated with NO overproduction.

Furthermore, the addition of this lignan maintained cell viability and induced a protective effect against glutamate-induced cell death in mouse hippocampal HT22 cells, suggesting neuroprotective activity (In et al., 2015).

(+)-pinoresinol has also been linked with hypoglycaemic agents because it inhibited rat intestinal α-glucosidase. This action is essential for the effective reduction of blood glucose levels and therefore could

play an important role in treating metabolic disorders, such as type 2 diabetes mellitus (T2DM) (Wikul et al., 2012).

4.3 Biological activity in vivo

There are few studies that have investigated the *in vivo* effects of pinoresinol, and those that have focused on very different areas. For example, in rats, this compound exhibited a dose-dependent protective effect against microvascular damage induced by hypoperfusion and reperfusion in pial circulation. Pinoresinol was able to induce vasodilation through reactive hyperaemia and reduce the generation of reactive oxygen species (ROS), protecting arteriolar wall integrity and capillary perfusion. Consequently, it also protected cerebral blood flow distribution and prevented brain injury (Lapi et al., 2015).

In mice, pinoresinol protected against CCL_4-induced acute hepatotoxicity and attenuated the release of transaminases ALT and AST, which is a process indicative of severe hepatocellular damage. These effects were supported by histological observations of liver sections. Pinoresinol also decreased inflammatory responses through the reduction of TNF-α, iNOS and COX-2 protein and mRNA expression, as well as the transactivation of NF-κβ and AP-1 (Kim et al., 2010).

Pinoresinol was also tested in *Caenorhabditis elegans* by Koch et al. (2015). Although these researchers detected noticeable radical scavenging activity in the ABTS assay, they did not detect any antioxidant effects in the nematodes *in vivo*. However, pinoresinol induced nuclear translocation of DAF-16, a pivotal transcription factor of the insulin/IGF-like signalling pathway that regulates the expression of protective genes such as superoxide dismutase.

Finally, a case-control study showed that high-level consumption of pinoresinol is associated with a significant reduction in breast cancer risk among premenopausal women (Torres-Sanchez, Galvan-Portillo, Wolff, & Lopez-Carrillo, 2009). However, dietary habits during the 12-month period prior to the onset of symptoms were only assessed in patients and control subjects after breast cancer was diagnosed, and no daily follow-up to the study was pursued, posing a limitation in the real value of pinoresinol consumption. Thus, more clinical trials are needed to confirm the relationship between the intake of pinoresinol and a low breast cancer risk.

Table 3 summarizes the *in vitro* and *in vivo* bioactivities associated with (+)-pinoresinol and 1-acetoxypinoresinol.

5. **Bioavailability, Absorption and Pharmacokinetics**

Although we are aware of many of the effects exhibited by pinoresinol and 1-acetoxypinoresinol, we must also consider whether the concentrations used in testing are similar to those naturally found in the organism after consumption. Several factor, including bioavailability (the tendency of the compound to be extracted from the food matrix) (Soler et al., 2010), metabolism by colonic bacteria and absorption by intestinal or target cells, must be taken into account. Little information has been published about these events with regards to 1-acetoxypinoresinol, although this compound has been shown to possess interesting biological activities.

5.1 Intake

The median intake of pinoresinol has been estimated for Dutch (Milder et al., 2005), French (Touillaud et al., 2007), Canadian (Cotterchio, Boucher, Kreiger, Mills, & Thompson, 2008) and Finnish (Nurmi, Mursu, Peñalvo, Poulsen, & Voutilainen, 2010) populations,

with values of 312, 422 , 107 and 442 μg/d, respectively. Plant lignans assessed in these studies were derived mainly from wholegrain and rye products, berries, coffee, tea, vegetables and fruit. Nevertheless, the daily intake of pinoresinol is expected to be much higher in regions with a Mediterranean diet, where consumption of olives and VOOs is higher. In addition, olives and VOOs are the only edible sources of 1-acetoxypinoresinol and, consequently, the only foods that could provide its potential health benefits.

5.2 Metabolism

Pinoresinol and 1-acetoxypinoresinol are quite stable under gastric and duodenal digestion conditions, as proven *in vitro* with models simulating both conditions (Soler et al., 2010). In fact, the concentrations of these compounds increased after digestion, perhaps due to the acidic conditions of the stomach, which are similar to those found during the oil extraction process. However, after ingestion, plant lignans are partly metabolized by human colonic bacteria before they are absorbed to form the mammalian lignans enterodiol and enterolactone (Heinonen et al., 2001). Conversion of pinoresinol to these enterolignans was approximately 55% in an *in vitro* metabolic model using human faecal microflora (Heinonen et al., 2001), and limited metabolism of phenols was also observed using human colon Caco-2/TC7 cells (Soler et al., 2010). In contrast, Nurmi et al. (2010) noted that pinoresinol should be extensively metabolized considering that only 5% of its intake was detected in the urine of Finnish men. In plasma samples, Suarez et al. (2009) quantified 1-acetoxypinoresinol at 60 and 120 min after the consumption of 30 mL of VOO, but they only found the native form (0.016 μM) in one of five subjects. However, the initial concentrations in the VOO used in the study are unknown, and it is difficult to attribute these results to the metabolism of the compound. On the other hand, the low number of subjects and the high variability observed among them make it impossible to reach conclusions about the concentrations reported.

In summary, metabolism of these plant lignans is not yet well characterized and varies between individuals depending on their diet, the source of the lignan, the expression of the metabolizing enzymes and the intestinal microflora, whose activity can vary from person to person and from day to day (Nurmi et al., 2010). Additionally, many other factors can affect circulating lignan levels in the body such as smoking, antibiotics, obesity (Adlercreutz, 2007) and the techniques used to analyse these levels, including the extraction method or time point of blood collection (Penalvo et al., 2004).

5.3 Bioavailability

Very little is known about the availability of (+)-pinoresinol and 1-acetoxypinoresinol in tissues or organs. However, a study carried out using oral administration of the tritium-labelled, dietary lignan secoisolariciresinol diglucoside (3H-SDG) could shed light on the accessibility of lignans to tumour cells, as tumour tissue radioactivity was up 92% in the serum of mice bearing MCF-7 breast cancer xenografts (Saarinen, Power, Chen, & Thompson, 2008). On the other hand, it is not known if these compounds exert their actions as a result of cellular internalization or if the mechanism of action could be reproduced outside of the cell through signalling pathways.

Owen et al. (2000c) proposed that because these lignans are fat soluble, a considerable proportion is likely to be absorbed and should exert effects on breast cancer and other diseases. Furthermore, the unabsorbed remainder will reach the large intestines where it can exert a chemopreventive effect against colorectal cancer.

Even though it has been shown that 55-73% of ingested olive oil phenols are absorbed (Vissers, Zock, Roodenburg, Leenen, & Katan, 2002), there is limited information about the cellular uptake of the compounds considered in the present review.

According to Garcia-Villalba et al. (2012), approximately 50% of the tested amount of pinoresinol was absorbed by JIMT-1 human breast cancer cells between 2 and 24 h after exposure. The absorption of 1-acetoxypinoresinol (approximately 40%) started within 1 h. In addition, During et al. (2012) showed that pinoresinol can be taken up by intestinal cells. After a 4 h exposure at a final concentration of approximately 40 μM, human colon adenocarcinoma Caco-2 cells took up 2% of the pinoresinol, from which 75% was in a conjugated form. The cellular uptake of free pinoresinol (6 to 259 μM) increased linearly with the initial concentration, indicating that it entered cells by simple diffusion or with the help of a low affinity transporter. In contrast, they showed that lignan conjugation is a saturable process, because conjugated forms first increased proportionally to the initial concentration and then plateaued at approximately 30-40 μM (During et al., 2012). The fact that a specific transporter is not required by cells for pinoresinol uptake indicates that this compound may be available for uptake by other cell types in which it could also exert its biological effects.

As reported by Sepporta et al. (2013), although plasma concentrations of lignans exist in the nanomolar range, tissue concentrations remain unknown and bioaccumulation phenomena cannot be excluded.

It is worth noting that the interaction between polyphenols and some food components such as proteins, carbohydrates, fibre, fat and alcohol, could affect their absorption, elimination and half-life in the blood (reviewed by D'Archivio, Filesi, Vari, Scazzocchio, & Masella, 2010). However, no references have discussed how these interactions or conjugation affect pinoresinol and 1-acetoxypinoresinol, and further investigation is needed.

6. Prospects

It has been reported that pinoresinol and 1-acetoxypinoresinol have several health benefit, from free radical scavenging activity to biological effects *in vitro* and *in vivo*. They have been associated with antitumor activity, especially in breast tumours, and with a decrease in the expression of proteins and molecules, such FASN and HER2 tyrosine kinase, that are directly implicated in cancer processes. Both compounds also possess chemical antioxidant activity. However, little data have been published about the effects of 1-acetoxypinoresinol and, taking into account the preliminary information known about this lignan, it would be worth further study.

References also suggest that (+)-pinoresinol exerts anti-inflammatory activity, specifically through the inhibition of NO but also through the inhibition of inflammatory mediators such as PGE2, COX-2, TNFα, IL1β, IL-6 and NF-κβ. Therefore, consumption of this compound could be clinically applied to target inflammatory processes, which are the basis for the initiation of several diseases such as cancer.

Furthermore, this compound has shown antifungal effects against several human pathogenic fungi, as well as neuroprotective and hypoglycaemic actions. Finally, pinoresinol's antioxidant activity, reported by a few authors who used DPPH and ABTS assays for analysis, seems to be effective both *in vitro* and *in vivo*.

Due to the potential pharmacological properties inherent within these lignans further investigation is needed in order to determine their effects and mechanisms of action. Moreover, it would be crucial to elucidate currently unknown aspects such as their concentrations in foods, their ability to be metabolized and whether cells or tissues can efficiently absorb them in their native form, including in what concentrations. However, it appears that these compounds remain stable during digestion, and there is a high probably that they enter

cells by simple diffusion, indicating their potential availability to nearly all tissues.

Thus, more studies are necessary in order to better understand these compounds, especially given that both (+)-pinoresinol and 1-acetoxypinoresinol have broad pharmacological potential as anti-tumour, anti-inflammatory, antioxidant and antifungal agents.

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Conflict of interests

The authors declare no competing financial or personal interest.

References

Adlercreutz, H. (2007). Lignans and human health. *Critical Reviews in Clinical Laboratory Sciences, 44*(5-6), 483-525. doi:783020201 [pii]

Antonini, E., Farina, A., Leone, A., Mazzara, E., Urbani, S., Selvaggini, R., . . . Ninfali, P. (2015). Phenolic compounds and quality parameters of family farming versus protected designation of origin (PDO) extra-virgin olive oils. *Journal of Food Composition and*

Analysis, 43, 75-81. doi:http://dx.doi.org/10.1016/j.jfca.2015.04.015

Arslan, D., Karabekir, Y., & Schreiner, M. (2013). Variations of phenolic compounds, fatty acids and some qualitative characteristics of sarıulak olive oil as induced by growing area. *Food Research International, 54*(2), 1897-1906. doi:http://dx.doi.org/10.1016/j.foodres.2013.06.016

Bonoli, M., Bendini, A., Cerretani, L., Lercker, G., & Toschi, T. G. (2004). Qualitative and semiquantitative analysis of phenolic compounds in extra virgin olive oils as a function of the ripening degree of olive fruits by different analytical techniques. *Journal of Agricultural and Food Chemistry, 52*(23), 7026-7032. doi:10.1021/jf048868m

Brenes, M., Hidalgo, F., Garcia, A., Rios, J., Garcia, P., Zamora, R., & Garrido, A. (2000). Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *Journal of the American Oil Chemists' Society, 77*(7), 715-720.

Brenes, M., Garcia, A., Dobarganes, M. C., Velasco, J., & Romero, C. (2002a). Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. *Journal of Agricultural and Food Chemistry, 50*(21), 5962-5967. doi:jf020506w [pii]

Brenes, M., Garcia, A., Garcia, P., & Garrido, A. (2001). Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. *Journal of Agricultural and Food Chemistry, 49*(11), 5609-5614. doi:jf0107860 [pii]

Brenes, M., García, A., Rios, J. J., García, P., & Garrido, A. (2002b). Use of 1-acetoxypinoresinol to authenticate picual olive oils.

International Journal of Food Science & Technology, 37(6), 615-625. doi:10.1046/j.1365-2621.2002.00591.x

Carpinella, M. C., Giorda, L. M., Ferrayoli, C. G., & Palacios, S. M. (2003). Antifungal effects of different organic extracts from melia azedarach L. on phytopathogenic fungi and their isolated active components. *Journal of Agricultural and Food Chemistry, 51*(9), 2506-2511. doi:10.1021/jf026083f

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Del Carlo, M., Gallina-Toschi, T., . . . Fernandez-Gutierrez, A. (2005). Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *Journal of Agricultural and Food Chemistry, 53*(23), 8918-8925. doi:10.1021/jf0515680

Chin, Y. W., Chai, H. B., Keller, W. J., & Kinghorn, A. D. (2008). Lignans and other constituents of the fruits of euterpe oleracea (acai) with antioxidant and cytoprotective activities. *Journal of Agricultural and Food Chemistry, 56*(17), 7759-7764. doi:10.1021/jf801792n

Chin, Y. W., Jones, W. P., Rachman, I., Riswan, S., Kardono, L. B., Chai, H. B., . . . Kinghorn, A. D. (2006). Cytotoxic lignans from the stems of helicteres hirsuta collected in indonesia. *Phytotherapy Research : PTR, 20*(1), 62-65. doi:10.1002/ptr.1806

Chiung, Y. M., Hayashi, H., Matsumoto, H., Otani, T., Yoshida, K., Huang, M. Y., . . . Nakayama, M. (1994). New metabolites, tetrahydrofuran lignans, produced by streptomyces sp. IT-44. *The Journal of Antibiotics, 47*(4), 487-491.

Chtourou, M., Gargouri, B., Jaber, H., Abdelhedi, R., & Bouaziz, M. (2013). Comparative study of olive oil quality from chemlali sfax

versus arbequina cultivated in tunisia. *European Journal of Lipid Science and Technology, 115*(6), 631-640. doi:10.1002/ejlt.201200234

Cotterchio, M., Boucher, B. A., Kreiger, N., Mills, C. A., & Thompson, L. U. (2008). Dietary phytoestrogen intake--lignans and isoflavones--and breast cancer risk (canada). *Cancer Causes & Control : CCC, 19*(3), 259-272. doi:10.1007/s10552-007-9089-2

Dar, A. A., & Arumugam, N. (2013). Lignans of sesame: Purification methods, biological activities and biosynthesis--a review. Bioorganic Chemistry, 50, 1-10. doi:10.1016/j.bioorg.2013.06.009

D'Archivio, M., Filesi, C., Vari, R., Scazzocchio, B., & Masella, R. (2010). Bioavailability of the polyphenols: Status and controversies. *International Journal of Molecular Sciences, 11*(4), 1321-1342. doi:10.3390/ijms11041321

During, A., Debouche, C., Raas, T., & Larondelle, Y. (2012). Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal caco-2 cells. *The Journal of Nutrition, 142*(10), 1798-1805. doi:jn.112.162453 [pii]

During, A., Debouche, C., Raas, T., & Larondelle, Y. (2012). Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal caco-2 cells. *The Journal of Nutrition, 142*(10), 1798-1805. doi:jn.112.162453 [pii]

Fini, L., Hotchkiss, E., Fogliano, V., Graziani, G., Romano, M., De Vol, E. B., . . . Ricciardiello, L. (2008). Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. *Carcinogenesis, 29*(1), 139-146. doi:bgm255 [pii]

Gambacorta, G., Faccia, M., Previtali, M. A., Pati, S., La Notte, E., & Baiano, A. (2010). Effects of olive maturation and stoning on quality indices and antioxidant content of extra virgin oils (cv. coratina) during storage. *Journal of Food Science, 75*(3), C229-35. doi:10.1111/j.1750-3841.2010.01516.x

García, A., Brenes, M., Martínez, F., Alba, J., García, P., & Garrido, A. (2001). High-performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale. *Journal of the American Oil Chemists' Society, 78*(6), 625-629.

Garcia-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Menendez, J. A., Segura-Carretero, A., & Fernandez-Gutierrez, A. (2012). Uptake and metabolism of olive oil polyphenols in human breast cancer cells using nano-liquid chromatography coupled to electrospray ionization-time of flight-mass spectrometry. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences, 898*, 69-77. doi:10.1016/j.jchromb.2012.04.021

Gómez Caravaca, A. M., Carrasco Pancorbo, A., Canabate Diaz, B., Segura Carretero, A., & Fernandez Gutierrez, A. (2005). Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil. *Electrophoresis, 26*(18), 3538-3551. doi:10.1002/elps.200500202

Harborne, J. B., Baxter, H., & Moss, G. P. (1999). *Phytochemical dictionary : A handbook of bioactive compounds from plants*. Wallington: Taylor & Francis.

Hashim, Y. Z., Rowland, I. R., McGlynn, H., Servili, M., Selvaggini, R., Taticchi, A., . . . Gill, C. I. (2008). Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in

vitro. *International Journal of Cancer.Journal International Du Cancer, 122*(3), 495-500. doi:10.1002/ijc.23148

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wahala, K., Deyama, T., . . . Adlercreutz, H. (2001). In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol. *Journal of Agricultural and Food Chemistry, 49*(7), 3178-3186. doi:jf010038a [pii]

Hirano, T., Fukuoka, K., Oka, K., Naito, T., Hosaka, K., Mitsuhashi, H., & Matsumoto, Y. (1990). Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. *Cancer Investigation, 8*(6), 595-602.

Hwang, B., Lee, J., Liu, Q. H., Woo, E. R., & Lee, D. G. (2010). Antifungal effect of (+)-pinoresinol isolated from sambucus williamsii. *Molecules (Basel, Switzerland), 15*(5), 3507-3516. doi:10.3390/molecules15053507; 10.3390/molecules15053507

In, S. J., Seo, K. H., Song, N. Y., Lee, D. S., Kim, Y. C., & Baek, N. I. (2015). Lignans and neolignans from the stems of vibrunum erosum and their neuroprotective and anti-inflammatory activity. *Archives of Pharmacal Research, 38*(1), 26-34. doi:10.1007/s12272-014-0358-9

Jung, H. W., Mahesh, R., Lee, J. G., Lee, S. H., Kim, Y. S., & Park, Y. K. (2010). Pinoresinol from the fruits of forsythia koreana inhibits inflammatory responses in LPS-activated microglia. *Neuroscience Letters, 480*(3), 215-220. doi:10.1016/j.neulet.2010.06.043

Kalua, C. M., Bedgood, D. R.,Jr, Bishop, A. G., & Prenzler, P. D. (2008). Changes in virgin olive oil quality during low-temperature

fruit storage. *Journal of Agricultural and Food Chemistry, 56*(7), 2415-2422. doi:10.1021/jf073027b

Kajla, P., Sharma, A., & Sood, D. R. (2015). Flaxseed-a potential functional food source. Journal of Food Science and Technology, 52(4), 1857-1871. doi:10.1007/s13197-014-1293-y

Kang, M. H., Naito, M., Sakai, K., Uchida, K., & Osawa, T. (2000). Mode of action of sesame lignans in protecting low-density lipoprotein against oxidative damage in vitro. *Life Sciences, 66*(2), 161-171. doi:S0024320599005743 [pii]

Kang, W., & Wang, J. (2010). In vitro antioxidant properties and in vivo lowering blood lipid of forsythia suspense leaves. *Med Chem Res, 19*, 617-628.

Kardono, L. B., Tsauri, S., Padmawinata, K., Pezzuto, J. M., & Kinghorn, A. D. (1990). Cytotoxic constituents of the bark of plumeria rubra collected in indonesia. *Journal of Natural Products, 53*(6), 1447-1455.

Kim, H. J., Ono, E., Morimoto, K., Yamagaki, T., Okazawa, A., Kobayashi, A., & Satake, H. (2009). Metabolic engineering of lignan biosynthesis in forsythia cell culture. *Plant & Cell Physiology, 50*(12), 2200-2209. doi:10.1093/pcp/pcp156

Kim, H. Y., Kim, J. K., Choi, J. H., Jung, J. Y., Oh, W. Y., Kim, D. C., . . . Lee, S. M. (2010). Hepatoprotective effect of pinoresinol on carbon tetrachloride-induced hepatic damage in mice. *Journal of Pharmacological Sciences, 112*(1), 105-112. doi:JST.JSTAGE/jphs/09234FP [pii]

Koch, K., Buchter, C., Havermann, S., & Watjen, W. (2015). The lignan pinoresinol induces nuclear translocation of DAF-16 in

caenorhabditis elegans but has no effect on life span. *Phytotherapy Research : PTR, 29*(6), 894-901. doi:10.1002/ptr.5330

Kulik, T., Busko, M., Pszczolkowska, A., Perkowski, J., & Okorski, A. (2014). Plant lignans inhibit growth and trichothecene biosynthesis in fusarium graminearum. *Letters in Applied Microbiology, 59*(1), 99-107. doi:10.1111/lam.12250

Kuo, P. C., Lin, M. C., Chen, G. F., Yiu, T. J., & Tzen, J. T. (2011). Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. *Journal of Agricultural and Food Chemistry, 59*(7), 3214-3219. doi:10.1021/jf104311g

Kwak, J. H., Kang, M. W., Roh, J. H., Choi, S. U., & Zee, O. P. (2009). Cytotoxic phenolic compounds from chionanthus retusus. *Archives of Pharmacal Research, 32*(12), 1681-1687. doi:10.1007/s12272-009-2203-0

Lapi, D., Di Maro, M., Mastantuono, T., Battiloro, L., Sabatino, L., Muscariello, E., & Colantuoni, A. (2015). Effects of oleuropein and pinoresinol on microvascular damage induced by hypoperfusion and reperfusion in rat pial circulation. *Microcirculation (New York, N.Y.: 1994), 22*(1), 79-90. doi:10.1111/micc.12175

Lee, J. W., Lee, J. H., Lee, C., Jin, Q., Lee, D., Kim, Y., . . . Hwang, B. Y. (2015). Inhibitory constituents of sophora tonkinensis on nitric oxide production in RAW 264.7 macrophages. *Bioorganic & Medicinal Chemistry Letters, 25*(4), 960-962. doi:10.1016/j.bmcl.2014.12.012

Li, C. Y., Zhai, H. Y., Tang, S. A., & Duan, H. Q. (2010). Studies on the chemical constituents of pachysandra terminalis and their

antioxidant activity. *Zhong Yao Cai = Zhongyaocai = Journal of Chinese Medicinal Materials, 33*(5), 729-732.

Lin, Y., Yngve, A., Lagergren, J., & Lu, Y. (2012). Dietary intake of lignans and risk of adenocarcinoma of the esophagus and gastroesophageal junction. *Cancer Causes & Control : CCC, 23*(6), 837-844. doi:10.1007/s10552-012-9952-7

Liu, J., Nakamura, S., Xu, B., Matsumoto, T., Ohta, T., Fujimoto, K., . . . Matsuda, H. (2015). Chemical structures of constituents from the flowers of osmanthus fragrans var. aurantiacus. *Journal of Natural Medicines, 69*(1), 135-141. doi:10.1007/s11418-014-0869-1

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*(5), 727-747.

Menendez, J. A., Vazquez-Martin, A., Garcia-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Fernandez-Gutierrez, A., & Segura-Carretero, A. (2008b). tabAnti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial extra-virgin olive oil (EVOO). *BMC Cancer, 8*, 377-2407-8-377. doi:10.1186/1471-2407-8-377

Menendez, J. A., Vazquez-Martin, A., Oliveras-Ferraros, C., Garcia-Villalba, R., Carrasco-Pancorbo, A., Fernandez-Gutierrez, A., & Segura-Carretero, A. (2008a). Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. *International Journal of Molecular Medicine, 22*(4), 433-439.

Menendez, J. A., Vazquez-Martin, A., Oliveras-Ferraros, C., Garcia-Villalba, R., Carrasco-Pancorbo, A., Fernandez-Gutierrez, A., &

Segura-Carretero, A. (2009). Extra-virgin olive oil polyphenols inhibit HER2 (erbB-2)-induced malignant transformation in human breast epithelial cells: Relationship between the chemical structures of extra-virgin olive oil secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2. *International Journal of Oncology, 34*(1), 43-51.

Milder, I. E., Feskens, E. J., Arts, I. C., Bueno de Mesquita, H. B., Hollman, P. C., & Kromhout, D. (2005). Intake of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in dutch men and women. *The Journal of Nutrition, 135*(5), 1202-1207. doi:135/5/1202 [pii]

Mimaki, Y., Kuroda, M., Asano, T., & Sashida, Y. (1999). Triterpene saponins and lignans from the roots of pulsatilla chinensis and their cytotoxic activity against HL-60 cells. *Journal of Natural Products, 62*(9), 1279-1283. doi:np9901837 [pii]

Minh, C. V., Nhiem, N. X., Yen, H. T., Kiem, P. V., Tai, B. H., Le Tuan Anh, H., . . . Kim, S. H. (2015). Chemical constituents of trichosanthes kirilowii and their cytotoxic activities. *Archives of Pharmacal Research, 38*(8), 1443-1448. doi:10.1007/s12272-014-0490-6

Nurmi, T., Mursu, J., Peñalvo, J. L., Poulsen, H. E., & Voutilainen, S. (2010). Dietary intake and urinary excretion of lignans in finnish men. *British Journal of Nutrition, 103*(05), 677-685. doi:10.1017/S0007114509992261

Oliveras López, M. J., Innocenti, M., Ieri, F., Giaccherini, C., Romani, A., & Mulinacci, N. (2008). HPLC/DAD/ESI/MS detection of lignans from spanish and italian olea europaea L. fruits. *Journal of Food Composition and Analysis, 21*(1), 62-70. doi:http://dx.doi.org/10.1016/j.jfca.2007.04.012

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B., & Bartsch, H. (2000a). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer (Oxford, England : 1990), 36*(10), 1235-1247. doi:S0959-8049(00)00103-9 [pii]

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Wurtele, G., Spiegelhalder, B., & Bartsch, H. (2000c). Olive-oil consumption and health: The possible role of antioxidants. *The Lancet.Oncology, 1*, 107-112.

Owen, R. W., Mier, W., Giacosa, A., Hull, W. E., Spiegelhalder, B., & Bartsch, H. (2000b). Identification of lignans as major components in the phenolic fraction of olive oil. *Clinical Chemistry, 46*(7), 976-988.

Pan, J. Y., Chen, S. L., Yang, M. H., Wu, J., Sinkkonen, J., & Zou, K. (2009). An update on lignans: Natural products and synthesis. Natural Product Reports, 26(10), 1251-1292. doi:10.1039/b910940d

Penalvo, J. L., Nurmi, T., Haajanen, K., Al-Maharik, N., Botting, N., & Adlercreutz, H. (2004). Determination of lignans in human plasma by liquid chromatography with coulometric electrode array detection. *Analytical Biochemistry, 332*(2), 384-393. doi:10.1016/j.ab.2004.05.046

Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., & McCullough, M. L. (2010). Dietary lignans: Physiology and potential for cardiovascular disease risk reduction. *Nutrition Reviews, 68*(10), 571-603. doi:10.1111/j.1753-4887.2010.00319.x

Rafieian-Kopaei, M., Setorki, M., Doudi, M., Baradaran, A., & Nasri, H. (2014). Atherosclerosis: Process, indicators, risk factors and new hopes. *International Journal of Preventive Medicine, 5*(8), 927-946.

Saarinen, N. M., Power, K. A., Chen, J., & Thompson, L. U. (2008). Lignans are accessible to human breast cancer xenografts in athymic mice. *Nutrition and Cancer, 60*(2), 245-250. doi:10.1080/01635580701649669

Saleem, M., Kim, H. J., Ali, M. S., & Lee, Y. S. (2005). An update on bioactive plant lignans. Natural Product Reports, 22(6), 696-716. doi:10.1039/b514045p

Selvaggini, R., Servili, M., Urbani, S., Esposto, S., Taticchi, A., & Montedoro, G. (2006). Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection. *Journal of Agricultural and Food Chemistry, 54*(8), 2832-2838. doi:10.1021/jf0527596

Sepporta, M. V., Mazza, T., Morozzi, G., & Fabiani, R. (2013). Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. *Nutrition and Cancer, 65*(8), 1208-1218. doi:10.1080/01635581.2013.828089

Servili, M., Taticchi, A., Esposito, S., Urbani, S., Selvaggini, R., & Montedoro, G. (2007). Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. *Journal of Agricultural and Food Chemistry, 55*(17), 7028-7035. doi:10.1021/jf070600i

Shi, Y. N., Shi, Y. M., Yang, L., Li, X. C., Zhao, J. H., Qu, Y., . . . Zhang, Y. J. (2015). Lignans and aromatic glycosides from piper wallichii and their antithrombotic activities. *Journal of Ethnopharmacology, 162*, 87-96. doi:10.1016/j.jep.2014.12.038

Sok, D. E., Cui, H. S., & Kim, M. R. (2009). Isolation and bioactivities of furfuran type lignan compounds from edible plants. *Recent Patents on Food, Nutrition & Agriculture, 1*(1), 87-95.

Soler, A., Romero, M. P., Macià, A., Saha, S., Furniss, C. S. M., Kroon, P. A., & Motilva, M. J. (2010). Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial caco-2/TC7 cell line. *Food Chemistry, 119*, 703-714.

Suarez, M., Romero, M. P., Macia, A., Valls, R. M., Fernandez, S., Sola, R., & Motilva, M. J. (2009). Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences, 877*(32), 4097-4106. doi:10.1016/j.jchromb.2009.10.025

Thompson, L. U., Chen, J. M., Li, T., Strasser-Weippl, K., & Goss, P. E. (2005). Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 11*(10), 3828-3835. doi:11/10/3828 [pii]

Toledo, E., Salas-Salvado, J., Donat-Vargas, C., Buil-Cosiales, P., Estruch, R., Ros, E., . . . Martinez-Gonzalez, M. A. (2015). Mediterranean diet and invasive breast cancer risk among women at high cardiovascular risk in the PREDIMED trial: A randomized clinical trial. *JAMA Internal Medicine, 175*(11), 1752-1760. doi:10.1001/jamainternmed.2015.4838

Torres-Sanchez, L., Galvan-Portillo, M., Wolff, M. S., & Lopez-Carrillo, L. (2009). Dietary consumption of phytochemicals and

breast cancer risk in mexican women. *Public Health Nutrition, 12*(6), 825-831. doi:10.1017/S136898000800325X

Touillaud, M. S., Thiebaut, A. C., Fournier, A., Niravong, M., Boutron-Ruault, M. C., & Clavel-Chapelon, F. (2007). Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. *Journal of the National Cancer Institute, 99*(6), 475-486. doi:99/6/475 [pii]

Tsukamoto, H., Hisada, S., & Nishibe, S. (1984). Lignans from bark of the olea plants. I. *Chemical & Pharmaceutical Bulletin, 32*(7), 2730-2735.

Umezawa, T. (2003). Diversity in lignan biosynthesis. *Phytochemistry Reviews, 2*(3), 371-390.

Vissers, M. N., Zock, P. L., Roodenburg, A. J., Leenen, R., & Katan, M. B. (2002). Olive oil phenols are absorbed in humans. *The Journal of Nutrition, 132*(3), 409-417.

Vuorela, S., Kreander, K., Karonen, M., Nieminen, R., Hamalainen, M., Galkin, A., . . . Heinonen, M. (2005). Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *Journal of Agricultural and Food Chemistry, 53*(15), 5922-5931. doi:10.1021/jf050554r

Wikul, A., Damsud, T., Kataoka, K., & Phuwapraisirisan, P. (2012). (+)-Pinoresinol is a putative hypoglycemic agent in defatted sesame (sesamum indicum) seeds though inhibiting alpha-glucosidase. *Bioorganic & Medicinal Chemistry Letters, 22*(16), 5215-5217. doi:10.1016/j.bmcl.2012.06.068

Yang, C. P., Huang, G. J., Huang, H. C., Chen, Y. C., Chang, C. I., Wang, S. Y., . . . Kuo, Y. H. (2013). The effect of the aerial part

of lindera akoensis on lipopolysaccharides (LPS)-induced nitric oxide production in RAW264.7 cells. *International Journal of Molecular Sciences, 14*(5), 9168-9181. doi:10.3390/ijms14059168

Yeo, H., Chin, Y. W., Park, S. Y., & Kim, J. (2004). Lignans of rosa multiflora roots. *Archives of Pharmacal Research, 27*(3), 287-290.

Yi, B., Hu, L., Mei, W., Zhou, K., Wang, H., Luo, Y., . . . Dai, H. (2011). Antioxidant phenolic compounds of cassava (manihot esculenta) from hainan. *Molecules (Basel, Switzerland), 16*(12), 10157-10167. doi:10.3390/molecules161210157

Figure 1. Chemical structures of (+)-pinoresinol and 1-acetoxypinoresinol.

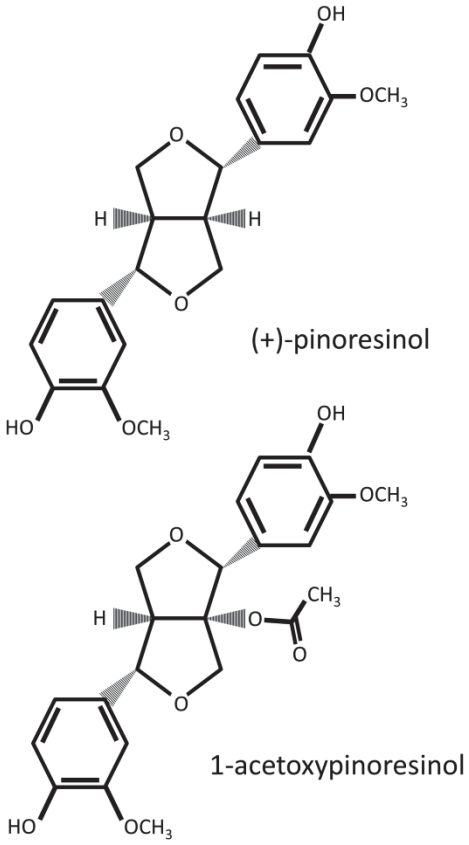

Table 1. Concentrations (mg/kg) of (+)-pinoresinol and 1-acetoxypinoresinol found in VOO obtained from different olive varieties. U = unspecified.

Olive variety	(+)-pinoresinol (mg/kg)	1-acetoxypinoresinol (mg/kg)	Reference
Arbequina	41.2 – 34	66.9 - 36.4	(Brenes et al., 2000)
	9.99 ± 0.32	36.2 ± 1.45	(Gomez Caravaca et al., 2005)
	2.83 ± 0.08	5.12 ± 0.95	(Chtourou, Gargouri, Jaber, Abdelhedi, & Bouaziz, 2013)
	46.2	77.3	(García et al., 2001)
Brisighella	12.35 ± 0.9	23.4 ± 1.2	(Antonini et al., 2015)
Chemlali Sfax	1.70 ± 0.03	6.23 ± 0.5	(Chtourou et al., 2013)
Coratina	U	11.32 - 6.88	(Gambacorta et al., 2010)
	8.4 ± 0.6	41.1 ± 1.1	(Servili et al., 2007)
Cornicabra	4.37 ± 1.33	1.2 ± 0.045	(Gomez Caravaca et al., 2005)
	74.5 ± 0.4	4.9 ± 0.2	(Selvaggini et al., 2006)
Empeltre	19 - 11.7	40 - 31.5	(Brenes et al., 2000)
Frantoio	55.1 ± 0.1	58.1 ± 0.1	(Selvaggini et al., 2006)
	17 ± 8	82 ± 8	(Kalua et al., 2008)
	4.2 ± 0.4	40.7 ± 2.3	(Servili et al., 2007)
Hojiblanca	30.8 - 24.4	30.5 - 3.7	(Brenes et al., 2000)
	6.97 ± 0.25	3.42 ± 0.14	(Gomez Caravaca et al., 2005)
	53.2 ± 0.4	78.2 ± 1.7	(Selvaggini et al., 2006)
Lechín de Granada	3.48 ± 0.13	0.86 ± 0.03	(Gomez Caravaca et al., 2005)
Lechín de Sevilla	0.62 ± 0.02	26.78 ± 0.99	(Gomez Caravaca et al., 2005)
Picual	36 - 29.5	4.9 - 2.7	(Brenes et al., 2000)
	6.94 ± 0.28	0.167 ± 0.006	(Gomez Caravaca et al., 2005)
	33.8	U	(García et al., 2001)
Picudo	31.2 - 29.1	12.5 - 6.8	(Brenes et al., 2000)

Table 2. Radical scavenging activity of (+)-pinoresinol and 1-acetoxypinoresinol.

Compound	Assay	IC_{50}	Reference
(+)-pinoresinol	DPPH	24.16 μM	(Kang & Wang, 2010)
		34.5 μM	(Kuo et al., 2011)
		96.82 μM	(Chin et al., 2008)
		558 μM	(Yi et al., 2011)
	ABTS	8.6 μM	(Kang & Wang, 2010)
		153.46 μM	(Yi et al., 2011)
		5 μM	(Koch et al., 2015)
1-acetoxypinoresinol	Hypoxanthine/xanthine oxidase	0.91 mM	(Owen et al., 2000a)

Table 3. Bioactivity of (+)-pinoresinol and 1-acetoxypinoresinol.

Compound	Activity	Form of action	Model	Reference
(+)-pinoresinol	Antitumor	FASN reduction	SKBR3 and MCF7 human breast cancer cells	(Menendez et al., 2008b)
		Decrease on HER2 protein expression and HER2 tyrosine kinase	MCF10A /HER2 human mammary epithelial cells	(Menendez et al., 2009)
		Cytotoxicity	MCF7 human breast cancer, LNCaP human prostate cancer, Lu1 and A549 human lung cancer, KB HeLa derivative, OK-OV-3 human ovarian cancer, MEL-2 human skin melanoma, HCT15 human colon cancer cells.	(Chin et al., 2006; Chiung et al., 1994; Kwak et al., 2009; Sepporta et al., 2013)
		Anti-proliferation, Induction of $p21^{WAF1/Cip1}$ expression	MDA-MB-231 human breast cancer cells, HL60 human leukemia cells	(Sepporta et al., 2013)
		Anti-invasiveness	HT115 human colon cancer cells	(Hashim et al., 2008)
		Reduction of breast cancer risk	Case-control study	(Torres-Sanchez et al., 2009)
	Antifungal	Inhibition of growth and trichothecene biosynthesis	*Fusarium verticilloides*, *Fusarium graminearum*, *Candida albicans*, *Trichosporon beigelii*, *Malassezia furfur*	(Hwang et al., 2010; Kulik et al., 2014)
	Antioxidant	Decrease of LDL and liposomes oxidation	*In vitro*	(Kang et al., 2000; Vuorela et al., 2005)
		Translocation of DAF-16	*C. elegans*	(Koch et al., 2015)
		ROS reduction	Male Wistar rats	(Lapi et al., 2015)

Table 3 (continuación)

	Anti-inflammatory	Inhibition of NO, PGE_2, COX-2, TNFα, IL1β, IL-6 and NF-κβ	J774 murine macrophages, Microglia of rats, Caco-2 human colon cancer cells, RAW264.7 murine macrophages	(During et al., 2012; In et al., 2015; Jung et al., 2010; Lee et al., 2015; Liu et al., 2015; Vuorela et al., 2005; Yang et al., 2013)
		Inhibition of TNFα, iNOS and COX-2. Transactivation of NF-κβ.	male ICR mice	(Kim et al., 2010)
	Neuroprotective	Cell viability and protective effect against induced cell death	HT22 mouse hippocampal cells	(In et al., 2015)
		Protection on microvascular damage induced, vasodilation.	Male Wistar rats	(Lapi et al., 2015)
	Hypoglycemic	Inhibition of α-glucosidase	Rat small intestine	(Wikul et al., 2012)
1-acetoxypinoresinol	Antitumor	FASN reduction	SKBR3 and MCF7 breast cancer cells	(Menendez et al., 2008)
		Decrease on HER2 protein and HER2 tyrosine kinase	MCF10A /HER2 mammary epithelial cells	(Menendez et al., 2009a)

4. Resumen global de resultados

1. Artículo 1: "**Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses**".

1.1 **Capacidad antioxidante del (+)-pinoresinol**

El compuesto mostró actividad antioxidante en los ensayos químicos basados tanto en la transferencia de electrones (ABTS y DPPH) como en la transferencia de átomos de hidrógeno (ORAC).

El radical $ABTS^{\bullet+}$ disminuyó con concentraciones de (+)-pinoresinol iguales o superiores a 10 μM tras probar un rango de entre 0.00001 y 1000 μM, siendo el efecto mayor que con el antioxidante estándar (Trolox). El radical DPPH también fue reducido por el (+)-pinoresinol, aunque en este caso con menor eficiencia que el antioxidante estándar (α-tocoferol). Para el ensayo ORAC, el (+)-pinoresinol mostró de nuevo una mayor protección que el Trolox.

1.2 **Influencia sobre la citotoxicidad y la proliferación**

Los resultados tras el tratamiento con 0.001, 0.01, 0.1, 1, 10 y 100 μM de (+)-pinoresinol mostraron efecto citotóxico a todas las concentraciones en MDA-MB-231, aunque las diferencias estadísticamente significativas se produjeron entre 0.001 y 1 μM, y a bajas concentraciones en MCF7. Esta tendencia se mantuvo en el ensayo de proliferación, donde la supervivencia celular fue inhibida en ambas líneas a 0.001, 0.01 y 0.1 μM. Cabe destacar que el efecto citotóxico y antiproliferativo fue más pronunciado en las células

que no expresan ERα (MDA-MB-231) que en las que sí expresan dicho receptor (MCF7).

Para las células no tumorales MCF10A, el (+)-pinoresinol produjo un efecto citotóxico menor que para los modelos tumorales, con una diferencia acusada para concentraciones entre 0.001 y 0.1 μM.

1.3 **Efectos sobre la apoptosis y el ciclo celular**

El tratamiento con (+)-pinoresinol no mostró cambios en el ciclo celular o inducción a apoptosis en las células estudiadas, salvo la concentración de 100 μM.

1.4 **Modulación del estrés oxidativo intracelular**

A través del ensayo con DCFH-DA conseguimos cuantificar la cantidad de EROs presentes en las células, tanto en condiciones basales como tras un choque oxidativo por la adición de H_2O_2, para simular el estrés oxidativo que se podría encontrar tanto en un estado de inducción del cáncer como en tumores ya establecidos.

En condiciones basales, el (+)-pinoresinol disminuyó los niveles de EROs de las células MCF10A de forma estadísticamente significativa a 10 y 100 μM, aunque esta tendencia se observó con todas las concentraciones usadas.

Bajo estrés oxidativo, los niveles de EROs en las células tumorales se incrementaron tras el tratamiento con (+)-pinoresinol, especialmente en MCF7, mientras que no se observó incremento en las células epiteliales no tumorales.

1.5 **Efectos sobre la integridad del ADN**

Para observar el efecto del (+)-pinoresinol en relación al estado del ADN, tanto en condiciones basales como induciendo daño a través de especies reactivas, se recurrió a la electroforesis unicelular alcalina o Comet Assay. El (+)-pinoresinol produjo daño al ADN en las células MCF7 tanto en estado basal como tras la adición de H_2O_2, mientras que en las células MDA-MB-231 solo fue capaz de aumentar dicho daño bajo condiciones basales. En las células no tumorales, previno el daño al ADN tras el choque oxidativo, aunque solo a 1 μM de forma estadísticamente significativa.

2. Artículo 2: "**The biological activities of natural lignans from olives and virgin olive oils: A review**".

2.1 **Actividad biológica de los lignanos (+)-pinoresinol y 1-acetoxypinoresinol**

Debido principalmente a la complejidad de obtención del 1-acetoxypinoresinol, los estudios sobre su actividad biológica son muy reducidos, aunque los pocos existentes le atribuyen propiedades farmacológicas como agente antioxidante y antitumoral.

Para el (+)-pinoresinol, varios autores han descrito actividad captadora de radicales libres a través de los ensayos ABTS y DPPH.

Los trabajos con modelos celulares vislumbran actividad antitumoral, antioxidante, antiinflamatoria, neuroprotectora e hipoglucémica del compuesto. Por ejemplo, como agente antitumoral, el (+)-pinoresinol actuó en células de mama (SKBR3, MCF7 y MCF10A) reduciendo la expresión de proteínas y moléculas directamente implicadas en procesos cancerosos, y en células de adenocarcinoma de colon (HT115) reduciendo su capacidad invasiva. También mostró efecto antiproliferativo en células de origen mamario (MDA-MB-231) y leucémicas (HL60) y efecto citotóxico en células tumorales de mama (MCF7), próstata (LNCaP), pulmón (Lu1 y A549), ovario (OK-OV-3), piel (MEL-2) y colon (HCT15). Como antiinflamatorio varios trabajos coinciden en la capacidad del (+)-pinoresinol para disminuir el NO en macrófagos J774 y RAW264.7, y otros mediadores proinflamatorios (PGE_2, COX-2, TNFα, IL1β, IL-6 y NFκβ) en células tumorales de colon (Caco-2) y microglía primaria de ratas.

La actividad *in vivo* del (+)-pinoresinol se ha estudiado en ratas Wistar, mostrando protección frente a daño microvascular y efecto vasodilatador, y en ratones ICR, donde de nuevo inhibió la expresión de TNFα, iNOX y COX-2. Finalmente, un estudio llevado a cabo en mujeres premenopáusicas asoció el consumo elevado de (+)-pinoresinol con la reducción en el riesgo de cáncer de mama.

2.2 **Consumo, metabolismo y biodisponibilidad**

El consumo medio de (+)-pinoresinol solo ha sido estimado en población holandesa (312 μg/día), francesa (422 μg/día), canadiense (107 μg/día) y finlandesa (442 μg/día), aunque es posible que dichas cantidades sean mayores en regiones con un elevado consumo de AOV.

El metabolismo del (+)-pinoresinol tampoco está bien caracterizado, aunque su estimación podría ser realmente difícil ya que depende de la dieta, la expresión de enzimas metabólicas y la microflora propias de cada persona, la fuente del lignano, etc. La conversión a enterolignanos tras su digestión fue del 55% en un modelo *in vitro*, mientras que solo el 5% de la cantidad de (+)-pinoresinol consumida se encontró en forma nativa en la orina de individuos finlandeses.

No obstante, parece ser que el (+)-pinoresinol se introduce en las células por difusión simple, sin necesidad de transportadores específicos, por lo que estaría disponible para cualquier tipo celular.

5. Discusión

Capacidad antioxidante química del lignano (+)-pinoresinol

Hasta la fecha, los pocos trabajos llevados a cabo sobre la capacidad antioxidante química del (+)-pinoresinol no parecen concluyentes, puesto que varían mucho en sus resultados, y en ningún caso utilizan el ensayo ORAC, considerado uno de los más relevantes desde el punto de vista biológico.

Según nuestros resultados, parece estar claro el papel antioxidante que el (+)-pinoresinol tiene per se, ya que en los 3 métodos químicos utilizados (ABTS, DPPH y ORAC) mostró actividad antioxidante de forma dosis-dependiente, y en ocasiones superior al antioxidante estándar utilizado en los ensayos.

Capacidad antitumoral del lignano (+)-pinoresinol

A pesar del papel preventivo frente al cáncer de mama que se les atribuye a los fitoestrógenos, apenas se ha investigado acerca de la acción quimiopreventiva o antitumoral que pudiera tener el fitoestrógeno (+)-pinoresinol, uno de los compuestos minoritarios más abundantes del AOV, en este tipo de cáncer.

En los estudios publicados hasta ahora, el (+)-pinoresinol ha mostrado actividad citotóxica en modelos experimentales celulares de diferentes tipos de cáncer, como próstata, pulmón, ovario o colon. Sin embargo, dicha actividad no se ha estudiado en las células tumorales de mama humana MDA-MB-231 y, aunque sí hay resultados en las células MCF7, no parecen estar claros, puesto que se limitan a un mismo autor que en un primer trabajo describió actividad citotóxica mientras que no encontró este efecto en un

trabajo posterior[117,118]. En relación a la posible actividad antiproliferativa, Sepporta *et al.*[119] encontraron que el (+)-pinoresinol inhibía el crecimiento de MDA-MB-231, pero no de MCF7. Sin embargo, su estudio se limitó a la concentración de 100 μM, la cual no podemos considerar como una concentración fisiológica.

En nuestro trabajo, llevado a cabo con rangos de concentraciones entre 0.001 y 100 μM, los resultados observados en las células tumorales de mama humana utilizadas sugieren un papel antitumoral del (+)-pinoresinol, ya que presentó capacidad citotóxica y antiproliferativa a bajas concentraciones tanto en las células altamente metastásicas (MDA-MB-231) como en las no metastásicas (MCF7).

Estos datos, a priori, podrían hacernos pensar que el compuesto, al ser un fitoestrógeno, tuviera actividad antagonista de estrógenos, es decir, que bloqueara los receptores estrogénicos provocando la regresión que se observa en muchos cánceres de mama dependientes de estas hormonas. Sin embargo, en nuestros ensayos no hemos utilizado suplementación con estrógenos, en cuyo caso estos mismos resultados permitirían atribuir el efecto antitumoral a

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[117] Chin YW, Jones WP, Rachman I, Riswan S, Kardono LB, Chai HB, et al. Cytotoxic lignans from the stems of Helicteres hirsuta collected in Indonesia. Phytother Res. 2006 Jan;20(1):62-5.

[118] Chin YW, Chai HB, Keller WJ, Kinghorn AD. Lignans and other constituents of the fruits of Euterpe oleracea (Acai) with antioxidant and cytoprotective activities. J Agric Food Chem. 2008 Sep 10;56(17):7759-64.

[119] Sepporta MV, Mazza T, Morozzi G, Fabiani R. Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. Nutr Cancer. 2013;65(8):1208-18.

la acción del compuesto como antagonista de estrógenos. Al no haber estrógenos en el medio, dicho efecto tiene que imputarse a otro mecanismo, que desconocemos actualmente.

Más aún, la actividad antitumoral se produjo con independencia de los niveles de expresión de receptores estrogénicos en dichas células, siendo el efecto citotóxico y antiproliferativo más pronunciado en las células que no expresan ERα (MDA-MB-231). De nuevo, no parece que estos efectos se deban a la interacción con este receptor.

Se puede descartar también que la actividad mostrada pueda ser debida a la activación del ERβ, ya que se conoce que ambos modelos celulares (y en general las células cancerosas de mama) presentan una baja expresión de este receptor. Además, este receptor ejerce efectos antiproliferativos en células cancerosas de mama positivas para ERα

Por tanto, y a pesar de que el (+)-pinoresinol es un fitoestrógeno, sus mecanismos de acción no parecen estar asociados a los receptores de estrógenos, como tampoco a efectos sobre el ciclo celular o la inducción a apoptosis, que no se vieron alterados en los ensayos. En 2008, Fini *et al.*[120] asumieron como probable que el (+)-pinoresinol actuara mediante la activación de la ruta ATM/p53, implicada en la reparación del ADN dañado, la detención del ciclo celular y la activación de apoptosis en caso necesario. Estos autores observaron que el tratamiento con extractos ricos en (+)-pinoresinol, en diferentes modelos celulares de cáncer de colon, inhibían la viabilidad celular, producían apoptosis y detenían el ciclo celular solamente en células con el gen p53 funcional. Sin embargo,

[120] Fini L, Hotchkiss E, Fogliano V, Graziani G, Romano M, De Vol EB, et al. Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. Carcinogenesis. 2008 Jan;29(1):139-46.

el hecho de trabajar con extractos impide atribuir estos efectos exclusivamente al (+)-pinoresinol, y el estudio posterior de Sepporta *et al.*[121], utilizando el compuesto puro sobre diferentes líneas celulares, mostró que el efecto antiproliferativo era independiente de la expresión de p53. Nuestros resultados también descartan la implicación de esta ruta, dada la ausencia de cambios en el ciclo celular y la inducción a apoptosis, y a que la viabilidad celular se redujo tanto en las células MDA-MB-231, deficientes para p53, como en MCF7, competentes para dicho gen. Estos datos abren la puerta a nuevas investigaciones encaminadas a conocer las vías de señalización implicadas en los efectos citotóxicos y antiproliferativos observados.

Por otra parte, el aumento de EROs por acción del (+)-pinoresinol sobre las células tumorales tras la adición de H_2O_2, plantea la posibilidad de considerar a este compuesto como adyuvante de terapias prooxidantes. Aprovechando el hecho de que las células cancerosas poseen niveles intracelulares de EROs más elevados que las no tumorales, muchos de los tratamientos quimioterápicos se basan en el incremento del estrés oxidativo por encima de un nivel no tolerable para las células tumorales, eliminándolas selectivamente. Además, el papel como posible adyuvante se ve reforzado por la acción diferencial del (+)-pinoresinol frente a las células epiteliales no tumorales en comparación con las tumorales, ya que las primeras no vieron incrementados sus niveles de EROs tras el choque oxidativo, sino que incluso hubo tendencia a disminuirlos, sugiriendo la ausencia de daño a las células epiteliales en el transcurso de las terapias encaminadas al aumento del estrés oxidativo hasta niveles tóxicos para las células tumorales.

[121] Sepporta MV, Mazza T, Morozzi G, Fabiani R. Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. Nutr Cancer. 2013;65(8):1208-18.

Las células MCF7 resultaron particularmente sensibles al aumento de las especies reactivas intracelulares tras el choque oxidativo, lo cual podría ser la causa del daño al ADN observado en estas células mediante el ensayo Comet.

Capacidad quimiopreventiva del lignano (+)-pinoresinol

Hasta ahora, nunca se habían descrito los efectos que causa el (+)-pinoresinol en relación a la viabilidad celular en células epiteliales de mama humana. Nuestros resultados de citotoxicidad y proliferación muestran un efecto citotóxico a bajas concentraciones bastante menor para las células no tumorales (MCF10A) en comparación con los modelos tumorales utilizados, lo que sugiere que el lignano posee acción diferencial, siendo capaz de ejercer efecto antitumoral con daño menor a tejidos no tumorales.

Además, la tendencia de este compuesto para disminuir el estrés oxidativo de las células epiteliales en condiciones basales, apunta hacia un papel preventivo en el desarrollo de cáncer. Puesto que niveles elevados de EROs pueden conducir al inicio, progresión y propagación del cáncer a través de daños al ADN o rutas de señalización protumorigénicas, la acción antioxidante ejercida por el (+)-pinoresinol le otorga propiedades quimiopreventivas.

A pesar de que este último efecto no se ve reflejado en el análisis de daño al ADN en condiciones basales, sí que se observa un efecto protector tras el choque oxidativo, lo que, por una parte, reafirma la ausencia de daño en tejido no tumoral en el posible papel como adyuvante de quimioterapias y, por otra, manifiesta la capacidad preventiva del (+)-pinoresinol, que podría inhibir el inicio y la progresión del cáncer de mama frente a ambientes protumorigénicos, mediante su capacidad para controlar los niveles de EROs intracelulares.

6. Conclusiones

- El compuesto (+)-pinoresinol posee actividad antioxidante química de forma dosis-dependiente, en base a los resultados de las técnicas ABTS, DPPH y ORAC.

- Se ha confirmado el efecto antitumoral del (+)-pinoresinol a bajas concentraciones, a través de su acción citotóxica, antiproliferativa y prooxidante en células tumorales de mama humanas, independientemente de los niveles de expresión de los receptores estrogénicos y de su grado de agresividad.

- Se han demostrado las características quimiopreventivas del (+)-pinoresinol en células epiteliales de mama humanas, en base a su efecto protector frente al daño al ADN, tras estrés oxidativo inducido, y a su actividad antioxidante.

- Los resultados obtenidos confirman que el (+)-pinoresinol, debido a las propiedades quimiopreventivas que ha mostrado *in vitro*, podría estar implicado en la baja incidencia de cáncer de mama asociada al consumo de aceite de oliva virgen. No obstante, ya que nuestros estudios se han realizado en modelos experimentales celulares, serían necesarios estudios adicionales *in vivo* para poder confirmar el potencial quimiopreventivo que este compuesto ha demostrado *in vitro*.

7. Anexos

Anexo I

Artículos publicados en revistas incluidas en el *Journal Citation Reports (Science Citation Index)*

López-Biedma, A., Sánchez-Quesada, C., Beltrán, G., Delgado-Rodríguez, M., Gaforio, J.J. Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses. *BMC Complement. Altern. Med.* 2016. 16: 350.

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BMC Complementary and Alternative Medicine

RESEARCH ARTICLE Open Access

Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses

Alicia López-Biedma[1], Cristina Sánchez-Quesada[1], Gabriel Beltrán[2], Miguel Delgado-Rodríguez[1,3] and José J. Gaforio[1*]

Abstract

Background: Consumption of virgin olive oil (VOO) has been associated with a low breast cancer incidence. Pinoresinol is a phytoestrogen that is typically found in VOO. Considering the role of oestrogen in breast cancer development and progression, we investigated the potential antitumor activity of pinoresinol in breast cancer cells.

Methods: To address this question, we treated MDA-MB-231 (oestrogen receptor [ER] negative) and MCF7 (ER+) human breast tumour cells and MCF10A human mammary epithelial cells (ER-) with different concentrations of pinoresinol. The cytotoxic activity, cell proliferation, cell cycle profile, apoptosis induction, reactive oxygen species production and DNA damage were assessed.

Results: Pinoresinol showed cytotoxic, anti-proliferative and pro-oxidant activity in human breast tumour cells, independent of their oestrogen receptor status. In addition, pinoresinol exerted antioxidant activity and prevented DNA damage associated with oxidative stress in human mammary epithelial cells.

Conclusions: Overall, the results suggest that pinoresinol may have antitumor activity in human breast cancer cells independently of oestrogen receptor status. Furthermore, the results show that the pinoresinol has the typical characteristics of a chemopreventive compound.

Keywords: Cytotoxic activity, Antioxidant, Virgin olive oil, Polyphenols, Chemopreventive

Background

Growing scientific evidence suggests that the intake of virgin olive oil (VOO), which is the main source of fat in Mediterranean diets, correlates with a low incidence of breast cancer [1]. Among the minor compounds present in VOO that possess different health properties [2–6], we find polyphenols to be a very interesting group because of their biological benefits. It has been reported that polyphenols prevent the development and progression of pathological conditions, such as cancer, neurological and cardio-vascular diseases, diabetes, aging, and so on [7].

One of the most abundant phenolic compounds in VOOs, behind tyrosol and hydroxytyrosol, is (+)-pinoresinol (PINO) [8] (Fig. 1). Its presence in VOOs depend on the variety of the cultivar, in amounts of 0.07 ± 0.003 mg/kg in Arbequina variety, about 0.90 ± 0.78 in Picual variety [9]. Brenes et al. [10] reported that Spanish olive oil contains a range of 20 to 45 mg/kg PINO. Several health properties have been attributed to PINO, including antifungal [11], anti-inflammatory [12, 13], hypoglycaemic [14] or chemopreventive biological activities [15, 16].

PINO has a chemical structure that is similar to that of oestrogen (i.e., it is a phytoestrogen). Oestrogen is essential for the growth and development of mammary glands and has been linked with the development and progression of breast cancer due to enhanced binding and activation of the oestrogen receptor α (ERα) [17]. For example, the phytoestrogen tamoxifen acts as an oestrogen antagonist in breast tissue and has been shown to slow breast cancer cell proliferation and has been used in clinical practice for breast cancer patients [18].

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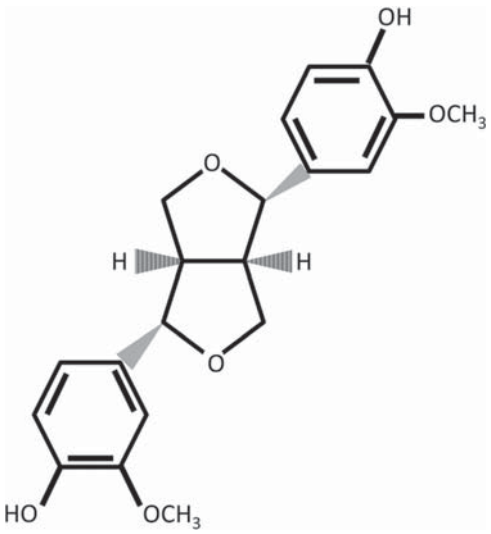

Fig. 1 Chemical structure of (+)-pinoresinol

Interestingly, ERβ has also been shown to mediate estrogenic action. The specific role of this receptor in human breast cancer remains elusive; however, in contrast to ERα, ERβ has been linked with anti-proliferative and pro-apoptotic activities. In fact, the expression of ERβ is lower in human breast cancer cells compared to normal breast cells, supporting its potential tumour-suppressive role [19].

Surprisingly, very few studies have noted the role of PINO as a potential agonist or antagonist of oestrogen and the chemopreventive repercussions that PINO treatment may have on hormone-related breast cancer [20].

The chemical antioxidant activity of PINO also remains unclear. A few studies using DPPH and ABTS assays have shown different antioxidant functions of PINO [21-23]. However, the Oxygen Radical Absorbance Capacity method (ORAC) has not been used in past studies, despite being considered one of the most biologically relevant assays [24].

Furthermore, the little research that has been done surrounding the effects of this compound on breast cancer cells remains inconclusive. Chin et al. [21] described a lack of cytotoxic effects and a cytoprotective effect of PINO on MCF7 cells stressed by H_2O_2 [25]. Other authors have reported anticancer effects of PINO by suppressing the expression of the lipogenic enzyme FASN in HER-2 overexpressing MCF7 cells [26]. Recently, Sepporta et al. [27] observed that PINO inhibited the growth of MDA-MB-231 cells, but not of MCF7 cells. Importantly, no previous study has examined the effects of PINO on a normal human breast cell line, which would address whether PINO plays a protective role against cancer development.

Therefore, the aim of the present study was to examine whether PINO exerts chemopreventive and/or antitumor activity in breast cancer, specifically because this compound is found in VOO and its consumption has been related with a minor incidence of breast cancer.

Therefore, to determine whether this compound may contribute, at least in part, to the health benefits attributed to VOO on breast cancer incidence and mortality, we studied the effects of PINO on breast cells with different receptor expression patterns. For this purpose, we used the following human mammary cells: highly invasive MDA-MB-231 (oestrogen receptor [ER] and progesterone receptor [PR] negative) breast tumour cells, the minimally invasive MCF7 (ER and PR positive) breast tumour cells and MCF10A human mammary epithelial cells (ER and PR negative).

Methods

Chemicals and material

The following were purchased from Gibco® Life Technologies Ltd (Paisley, UK): HuMEC Ready Medium (1X), TrypLE™ Express Enzyme (1X) and Minimum Essential Medium (MEM). Foetal bovine serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, Austria). Ethanol 96 % v/v and potassium peroxodisulfate ($K_2S_2O_8$) (CAS 7727-21-1) were purchased from Panreac Química S.L.U. (Barcelona, Spain). The CellTiter-Blue® Cell Viability Assay was acquired from Promega Corporation (Madison, WI, USA). Round bottom culture plates and cell culture flasks were purchased from Nunc A/S (Roskilde, Denmark). Flat bottom culture plates were from CytoOne (Hamburg, Germany). Fluorescein (FL) (CAS 2321-07-5) was obtained from Life Technologies (Carlsbad, CA, USA). The following were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA): (+)-Pinoresinol (CAS 487-36-5) purity ≥95 %; PBS; (S)-(+)-camptothecin (CPT) (CAS 7689-03-4) purity ≥90 %; 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (CAS 4091-99-0) purity ≥97 %; Sodium pyruvate solution (CAS 113-24-6); MEM Non-essential Amino Acid Solution (NEAA); HEPES buffer solution (CAS 7365-45-9); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (CAS 1898-66-4); 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (CAS 30931-67-0) purity ≥98 %; (±)-α-Tocopherol (Vitamin E) (CAS 10191-41-0) purity ≥96 %; (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox™) (CAS 53188-07-1) purity 97 % and 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) (CAS 2997-92-4) purity 97 %. PBS (1X, Dulbecco's) and DMSO (CAS 67-68-5) were obtained from AppliChem GmbH (Darmstadt,

Germany). The PI/RNase Staining Buffer kit, Annexin V-FITC kit and Comet Assay kit (CAS 50-07-7) were purchased, respectively, from BD Biosciences, Pharmingen (San Diego, CA), Miltenyi Biotec (Bergisch Gladbach, Germany) and Trevigen, Inc. (Gaithersburg, MD, USA). Non-tumorigenic human breast epithelial cells (MCF10A), minimally invasive human breast cancer cells (MCF7) and highly invasive human breast cancer cells (MDA-MB-231) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

ABTS radical scavenging test

The ABTS radical scavenging activity was measured as previously reported [28]. ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting 7 mM ABTS with 2.45 mM $K_2S_2O_8$ (final concentration) for 16 h in the dark at room temperature. The radical obtained was diluted in ultrapure water until the absorbance at 734 nm was between 0.7 and 1. Ethanol solutions of Trolox™ (antioxidant standard) or pinoresinol (PINO) were diluted in ultrapure water to reach concentrations between 50 and 800 μM and 0.00001 and 1000 μM, respectively. Twenty microliters of each concentration of Trolox™, PINO, ultrapure water (blank) or ethanol control (10 %) were added into a flat bottom 96-well plate. The reaction was initiated by the addition of 50 μL of $ABTS^{\bullet+}$, and the absorbance at 734 nm was immediately measured every 5 min over 2 h at 30°C with a TECAN GENios Plus microplate reader (Tecan Group Ltd., Switzerland). All of the reactions were performed in triplicate, in three independent experiments. The percentage of the radical scavenging activity (% RSA) was calculated according to the following formula:

$$\%\,\mathrm{RSA} = 100\left(A_{C(0)} - A_{A(t)}\right)/A_{C(0)} \quad (1)$$

where $A_{C(0)}$ is the absorbance of the blank at t = 0 and $A_{A(t)}$ is the absorbance of the compound/standard at t = 60.

Radical scavenging activity by the DPPH assay

Estimation of the antioxidant capacity against the radical DPPH was carried out according to Brand-William et al., [29] with some modifications. An ethanolic solution of DPPH 100 μM (final concentration) was mixed in 96-well plates with ethanolic solutions of PINO or α-tocopherol (antioxidant standard) at 0.03, 0.06, 0.13, 0.25, 0.5, 1 and 2 mole ratios (moles of antioxidant/moles of DPPH). DPPH samples without antioxidants were also measured as blank controls. The absorbance at 520 nm was read every 5 min over 2 h with a TECAN GENios Plus microplate reader. Measurements were performed at least in triplicate in three separate experiments. The radical scavenging activity (% RSA) was calculated as described in Eq. (1) (t = 60).

$ORAC_{FL}$ assay

The Oxygen Radical Absorbance Capacity (ORAC) of PINO was assayed as described in Prior et al. [30]. This method measures the oxidative degradation of fluorescein induced by the thermal decomposition of the AAPH azo-compound. In brief, fluorescein (48 nM) was added to each well of a round bottom 96-well plate that was previously tempered at 37°C. Then, PINO (from 0.001 to 1000 μM), Trolox™ (standard, from 12.5 to 100 μM) or PBS (blank) with a final volume of 1 % DMSO (v/v) was added to the wells. After incubating for 15 min at 37 °C, AAPH was added to the wells. Fluorescence readings (Ex. λ_{485}/Em. λ_{520} nm) were taken every 5 min at 37 °C for 160 min with a TECAN GENios Plus microplate reader. The 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 determined as follows:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_n/f_0 \quad (2)$$

where f_0 is the initial fluorescence at cycle 0 and f_n is the fluorescence reading at cycle n.

The results were expressed as micromolar Trolox™ equivalents (TE), which were calculated using the line equation from the standard curve:

$$TE = (Y - b)/m \quad (3)$$

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

Cell culture and treatments

Human MCF10A (ERα and PR negative) breast epithelial cells were grown in HuMEC Ready Medium. Human MCF7 (ERα and PR positive) and MDA-MB-231 (ERα and PR negative) breast cancer cells were grown in MEM supplemented with 10 % FBS, 1 % Hepes buffer, 1 % NEAA and 1 % Sodium Pyruvate. The cells were cultivated as monolayer cultures in a humidified atmosphere with 5 % CO_2 at 37°C and subcultured using TryPLE Express. Cells growing between 90 and 95 % of confluence were used for all experiments. The cells were treated for 24 h with 0.001, 0.01, 0.1, 1, 10 and 100 μM of PINO that was previously dissolved in DMSO (stock concentration 50 mM).

Cytotoxicity assay

The effects of PINO on cell viability were determined by the CellTiter-Blue® Cell Viability Assay according to the manufacturer's protocol with some modifications. A total of $5x10^3$ cells/well (for MDA-MB-231 and MCF7)

or 2.5x10^3 cells/well (for MCF10A) were seeded onto a 96-well plate. After 24 h to allow for cell attachment, the cells were treated with PINO or DMSO (as vehicle control) for another 24 h. CellTiter-Blue® was then added, and the plates were incubated for 3 h in darkness at 5 % CO_2 and 37°C. Finally, fluorescence was read with a TECAN GENios Plus microplate reader (Ex. λ_{485}/Em. λ_{595} nm) and viability was calculated using the formula:

$$\%\ viable\ cells\ =\ [(A_{treated\ cells})/A_{control}]\ x\ 100 \qquad (4)$$

where A corresponds to the relative fluorescence units of each sample. All of the measurements were performed in triplicate and each experiment was repeated at least three independent times.

Cell proliferation assay

In all of the cell proliferation experiments performed, the cells were seeded cells onto 96-well plates and allowed to attach before adding PINO or DMSO as the vehicle control. After 24 h of treatments, the medium was replaced by fresh medium and the plates were incubated for another 24 h. Then, CellTiter-Blue® was added, and fluorescence was read after 3 h of incubation with a TECAN GENios Plus microplate reader (Ex. λ_{485}/Em. λ_{595} nm). The measurements were repeated at 48, 72 and 96 h. The percentage of viable cells was calculated as defined in Eq. (4).

Cell cycle analysis

A total of 1 x 10^5 cells/mL (for MDA-MB-231 and MCF7 cells) or 5 x 10^4 cells/mL (for MCF10A cells) were seeded and allowed to attach for 24 h before treating with PINO for another 24 h. The cells were then fixed in cold 70 % ethanol, stored at −20°C for at least 24 h and labelled with a PI/RNase Staining Buffer kit. Cell cycle assessment was conducted by flow cytometry in an EPICS XL-MLC flow cytometer (Beckman Coulter, Spain), and the results were analysed using the FlowJo program (v5.7.2). Each experiment was repeated three independent times.

Apoptosis analysis

MDA-MB-231 (1 x 10^5 cells/mL), MCF7 (1 x 10^5 cells/mL) or MCF10A (5 x 10^4 cells/mL) cells were seeded, allowed to attach and treated for 24 h with PINO. The cells and supernatants were collected and labelled with Annexin V-FITC kit according to the manufacturer's suggestions. As a positive control, the cells were incubated with 1 μM camptothecin (CPT). Apoptosis analysis was carried out using an EPICS XL-MLC flow cytometer, and the results were analysed using the FlowJo program. Each experiment was repeated three independent times.

Detection of reactive oxygen species

Detection of intracellular Reactive Oxygen Species (ROS) was performed using the probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as previously reported by our group [31]. In brief, MCF10A (5.5x10^3 cells/well), MDA-MB-231 or MCF7 cells (7x10^3 cells/well) were seeded onto 96-well plates, allowed to attach for 24 h and then treated with PINO for an additional 24 h. After the addition of DCFH-DA (100 μM), the plates were incubated for 30 min at 37 °C and 5 % CO_2. Fluorescence was then read for 30 min (Ex. λ_{485}/Em. λ_{535}) with a TECAN GENios Plus microplate reader.

It is well known that the addition of H_2O_2 increases stress in culture cells [32]. To test whether PINO had a protective role against induced oxidative stress, the assay was also performed after the addition of H_2O_2 (400 μM) 30 min before quantification.

Both experimental conditions were assayed three independent times, and each measurement was performed in quadruplicate. In all cases, iron free media (MEM or HuMEC) were used.

The intracellular ROS level percentage was calculated as follows:

$$F\ =\ [(Ft_{30}–Ft_0)/Ft_0]\ x\ 100 \qquad (5)$$

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

Alkaline single-cell gel electrophoresis (Comet assay)

To estimate the state and wholeness of DNA, 5x10^4 cells/well (for MCF10A cells) or 1x10^5 cells/well (for MCF7 and MDA-MB-231 cells) were allowed to attach to a 12-well plate and treated with increasing PINO concentrations for 24 h. The cells were then detached and centrifuged twice in PBS. To evaluate whether PINO had the ability to protect against oxidative DNA damage, cells were also exposed to H_2O_2. The comet assay was carried out according to Warleta et al. [3]. Analysis of the DNA strands was performed by examining twenty-five random cell images per sample in a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss; Jena, Germany) equipped with Luca EMCCD camera (Andor Technology, Belfast, UK) and using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). DNA damage was calculated by determining the relative fluorescence between the head and tail using the olive tail moment (Olive_TM), which was defined as:

$$Olive_TM\ =\ [(tail\ (mean)\ –\ head\ (mean))\ x\ tail\ (\%\ DNA)]/100 \qquad (6)$$

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test

with the STATGRAPHICS Centurion XVI software (Statpoint Technologies, Inc. Warrenton, VA, USA). The values of $p < 0.05$ were considered significant. The data are represented as the mean of at least three independent experiments ± SEM and are expressed relative to the untreated controls.

Results

ABTS radical scavenging test

The radical cation $ABTS^{\bullet+}$ was diminished by pinoresinol (PINO) above 10 μM (Fig. 2), and the studied range was from 0.00001 to 1000 μM. Concentrations lower than 10 μM did not shown antioxidant capacity (data not shown). The antioxidant effects of PINO were higher than the antioxidant standard, as the 50 % of Radical Scavenging Activity (RSA) occurred at 380 μM for Trolox™ and at 274 μM for PINO.

Radical scavenging activity by the DPPH assay

As depicted in Fig. 3, PINO exhibited antioxidant activity against the DPPH radical in a dose dependent manner. The RSA of PINO was determined to be 50 % at 0.69 mol ratio

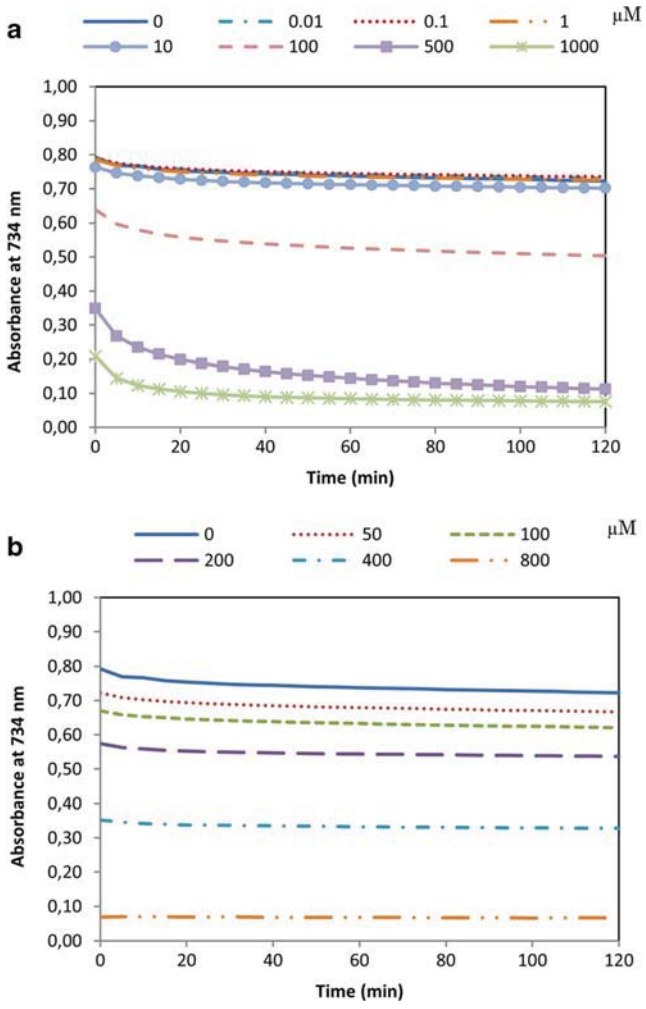

Fig. 2 ABTS radical scavenging assay. $ABTS^{\bullet+}$ radical scavenging activity of (+)-pinoresinol **a** or Trolox™ **b** at concentrations between 0.01and 1000 μM and 50 and 800 μM, respectively. Data are expressed as the absorbance of $ABTS^{\bullet+}$, which was read at 734 nm for 2 h. Measurements were performed in triplicate in three independent experiments

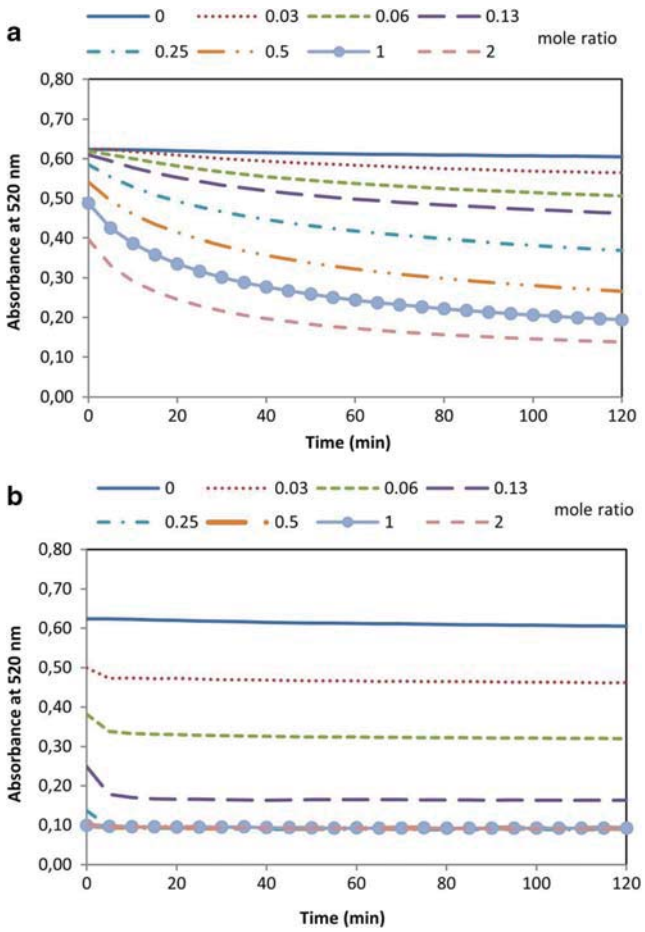

Fig. 3 DPPH radical scavenging assay. Antioxidant activity of (+)-pinoresinol **a** against DPPH radicals at 0.03, 0.06, 0.13, 0.25, 0.5, 1 and 2 mole ratios (mol antioxidant/mol DPPH). α-tocopherol **b** was used as the antioxidant control at the same ratios. Data represents the absorbance of DPPH radicals read at 520 nm for 2 h. Each experiment was performed in triplicate and repeated three times

(69 μM), while the RSA of the antioxidant control α-tocopherol was 50 % at 0.11 mol ratio (11 μM).

$ORAC_{FL}$ assay

The peroxyl radical scavenging activity of PINO, as measured by $ORAC_{FL}$, showed a protective effect against AAPH-induced peroxyl radical activity. PINO exerted a higher protection than Trolox™. The micromolar Trolox™ equivalents (TE) values were 39.95, 64.93, 114.89 and 214.81 for 12.5, 25, 50 and 100 μM of PINO.

Cytotoxicity assay

To assess the potential cytotoxic effects of PINO, MDA-MB-231, MCF7 and MCF10A cells were treated with concentrations of PINO ranging from 0.001 to 100 μM for 24 h. Surprisingly, PINO treatment was shown to promote a widespread cytotoxic effect at low concentrations in MCF7 cells and at all of the concentrations tested in MDA-MB-231 cells, although statistically significant changes were only observed from 0.001 to 1 μM (Fig. 4). Importantly, the percentage of non-tumorigenic human mammary epithelial cells death following treatment with 0.001 μM PINO was much lower (10 %) than in breast cancer cells (29 % for MDA-MB-231 and 20 % for MCF7 cells). In addition, a 10 μM PINO dose was shown to inhibit proliferation in MCF7 cells but did not induce significant cytotoxicity in MCF10A. Interestingly, a statistically significant cytotoxic effect was observed following

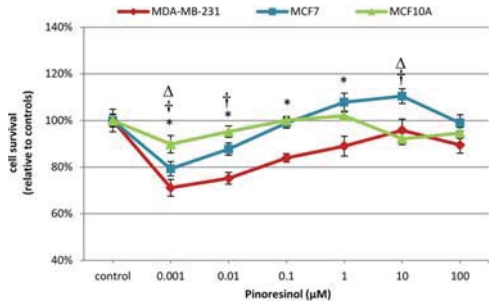

Fig. 4 Cytotoxicity assay. Cell survival measured by CellTiter-Blue® after 24 h of (+)-pinoresinol treatment on MDA-MB-231, MCF7 and MCF10A cells. Data are represented as the treatment average (±SEM) with respect to the control, which was considered as 100 %, for three independent assays carried out in triplicate. * MDA-MB-231, † MCF7 and Δ MCF10A indicate statistically significant differences at $p < 0.05$

0.01 μM PINO treatment in both types of human breast tumour cells tested, but not in human mammary epithelial cells.

Cell proliferation

Proliferation of MDA-MB-231 (Fig. 5a), MCF7 (Fig. 5b) and MCF10A cells (Fig. 5c) was determined after treatment with PINO for 24 h followed by incubation with fresh medium. Measurements were performed at 24, 48, 72 and 96 h following treatment removal. At 0.001, 0.01 and 0.1 μM, cell survival was inhibited in MDA-MB-231 and MCF7 cells. Surprisingly, at 0.001 μM, proliferation was reduced in tumour cells, but not in mammary epithelial cells, in a statistically significant manner. Strong proliferation was observed in MCF10A cells treated with up to 100 μM PINO, whereas neither MDA-MB-231 nor MCF7 showed this effect.

Analysis of cell cycle and apoptosis

PINO treatment did not produce cell cycle alterations in the three cell lines studied, with the exception of the 100 μM concentration, which diminished the percentage of cells in the S phase in a statistically significant manner (Table 1). This percentage was 8.34 ± 0.94 vs. 17.01 ± 2.33 of the control for MDA-MB-231 (a decrease of 50.97 % respect to the control), 11.96 ± 0.68 vs. 16.72 ± 0.86 for MCF7 (decrease of 28.47 %) and 9.92 ± 1.02 vs. 20.96 ± 1.29 for MCF10A (decrease of 52.67 %). PINO also increased the percentage of cells in G0/G1 for MDA-MB-231 (73.39 ± 1.69 vs. 60.67 ± 4.8 of the control, i.e., increase of 20.97 %) and MCF10A cells (76.1 ± 2.01 vs. 56.45 ± 0.2, that is, 34.81 % of increase respect to the control) and decreased the percentage of cells in 47.24 % in the G2/M phase in MCF10A cells (9.45 ± 2.1 vs. 17.91 ± 1.08 of the control). Unfortunately, 100 μM concentrations are not considered to be physiologically relevant. Representative cell cycle histograms of MDA-MB-231, MCF7 and MCF10A cells treated with PINO are shown in Additional file 1.

Statistically significant levels of apoptosis were induced in MCF10A cells treated with 100 μM PINO, with an increment of 445.86 % respect to the control (7.26 ± 2.54 vs. 1.33 ± 0.42) (Table 2). An increase of 19.72 % in apoptosis and 42.98 % in cell death, albeit not statistically significant, also appeared in MDA-MB-231 cells treated at this concentration (18.46 ± 5.92 vs. 14.82 ± 4.76 and 3.42 ± 1.09 vs. 1.95 ± 0.6, respectively). No significant pro-apoptotic effects of PINO were reported in MCF7 cells. Additional file 2 represents the flow cytometry analysis of MDA-MB-231, MCF7 and MCF10A cells after treatment with PINO.

DCFH-DA

Reactive Oxygen Species (ROS) were measured using the DCFH-DA method under basal conditions (Fig. 6a) and after oxidative stress induced by H_2O_2 (Fig. 6b). In the basal state, 10 and 100 μM concentrations of PINO were shown to diminish ROS levels in a statistically significant way in MCF10A and MDA-MB-231 cells, whereas all concentrations of PINO decreased ROS levels in MCF10A cells (Fig. 6a). Under conditions of oxidative stress (Fig. 6b), the presence of ROS was increased in breast cancer cell lines, especially MCF7 cells, with statistically significant levels observed at 1, 10 and 100 μM in MCF7 cells and at 100 μM in MDA-MB-231 cells. Importantly, increased ROS production was not observed in PINO-treated human mammary epithelial cells (MCF10A).

Comet assay

The percentage of DNA damage was determined by alkaline single-cell gel electrophoresis and expressed as Olive_TM. Data were expressed as the percentage relative to the basal (untreated) control, which was set as 100 %. For MDA-MB-231 cells (Fig. 7a), DNA was injured by PINO under basal conditions at 0.1, 1 and 100 μM, but was protected after additional stress with H_2O_2. For MCF7 cells (Fig. 7b), treatment with PINO tended to increase DNA damage with respect to both the untreated control and H_2O_2-treated control; however, statistically significant changes were only observed at 100 μM. Finally, PINO treatment was shown to have more of a protective effect in MCF10A cells (Fig. 7c) treated with H_2O_2 compared to the basal state. Indeed, a statistically significant reduction in DNA damage (93 %) was observed at 1 μM. Fig. 7d shows representative comet assay images of MDA-MB-231, MCF7 and MCF10A cells under different conditions.

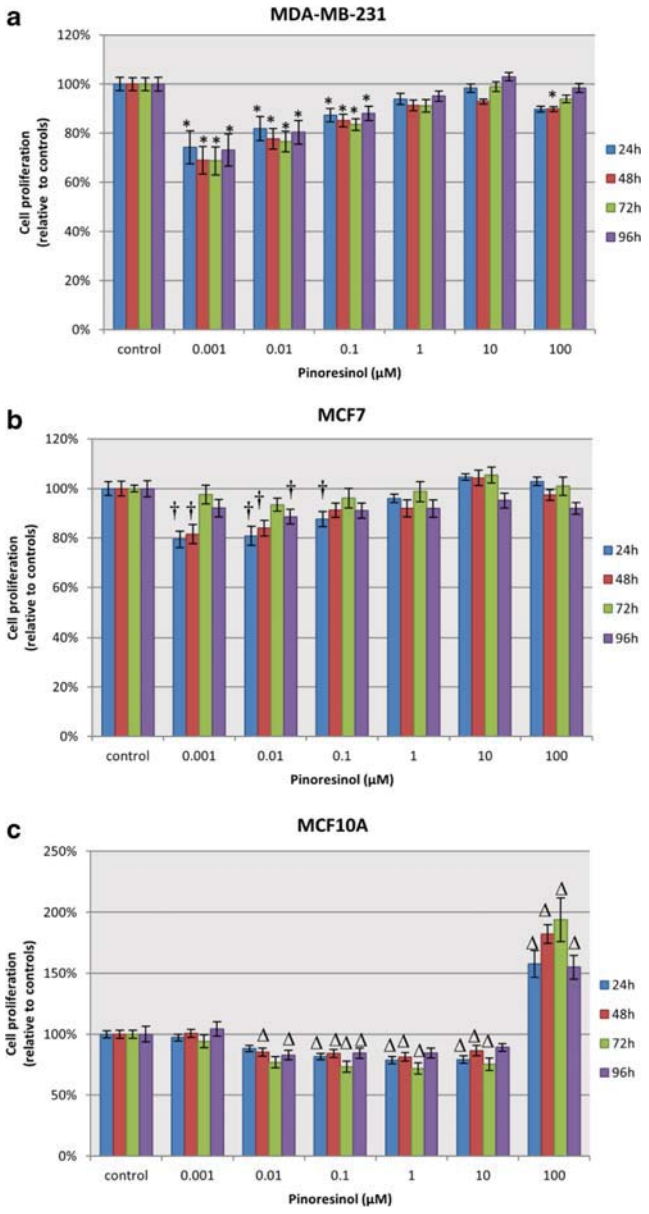

Fig. 5 Cell proliferation. Cell proliferation was measured by CellTiter-Blue® after 24 h of (+)-pinoresinol treatment followed by proliferation periods of 24, 48, 72 and 96 h in MDA-MB-231 (**a**), MCF7 (**b**) and MCF10A (**c**) cells. Data are represented as the mean (±SEM) with respect to the controls, which were set as 100 %, for three independent assays carried out in triplicate. *, † and Δ denote statistically significant differences relative to the control at $p < 0.05$ for MDA-MB-231, MCF7 or MCF10A, respectively

Discussion

As early as 1980s, it was suggested that lignans might prevent breast cancer and that this effect might be correlated with their phytoestrogenic activity. In addition, consumption of VOO, which contains significant amounts of lignans (e.g., PINO and 1-acetoxypinoresinol) as the major

Table 1 Percentage of cell cycle phases was measured by flow cytometry after treatment with PINO

MDA-MB-231					MCF7				MCF10A				
	SubG0/G1	G0/G1	S	G2/M	SubG0/G1	G0/G1	S	G2/M	SubG0/G1	G0/G1	S	G2/M
Control	1.72 ± 0.3	60.67 ± 4.8	17.01 ± 2.33	20.59 ± 2.46	0.69 ± 0.11	61.57 ± 0.19	16.72 ± 0.86	21.00 ± 0.74	1.23 ± 0.17	56.45 ± 0.2	20.96 ± 1.29	17.91 ± 1.08
0.001 μM	2.57 ± 0.7	60.87 ± 3.37	16.75 ± 1.68	20.23 ± 2.56	0.54 ± 0.03	62.71 ± 0.23	16.70 ± 0.43	20.60 ± 0.73	1.86 ± 0.7	55.32 ± 1.43	22.44 ± 0.34	16.77 ± 1.02
0.01 μM	1.80 ± 0.21	59.16 ± 4.53	17.55 ± 2.68	20.71 ± 2.01	0.62 ± 0.14	61.64 ± 1	16.72 ± 0.25	21.58 ± 0.93	1.67 ± 0.42	54.12 ± 2.3	21.79 ± 0.62	17.36 ± 1.47
0.1 μM	1.32 ± 0.24	59.33 ± 4.15	18.92 ± 1.68	20.35 ± 2.38	0.49 ± 0.02	60.71 ± 0.51	18.41 ± 0.5	20.61 ± 0.64	1.45 ± 0.22	54.84 ± 1.19	20.47 ± 0.53	19.09 ± 1.1
1 μM	1.62 ± 0.29	60.63 ± 4.93	18.56 ± 2.45	19.37 ± 2.48	0.49 ± 0.03	62.99 ± 1.28	16.99 ± 0.62	20.12 ± 1.06	1.85 ± 0.25	54.72 ± 0.65	21.23 ± 0.8	17.58 ± 1.16
10 μM	1.76 ± 0.3	64.85 ± 4.74	17.6 ± 2.38	15.52 ± 2.21	0.57 ± 0.03	61.73 ± 0.65	18.08 ± 0.41	19.62 ± 0.73	1.66 ± 0.12	53.93 ± 1.54	20.23 ± 1.1	19.6 ± 0.84
100 μM	2.29 ± 0.44	73.39 ± 1.69[a]	8.34 ± 0.94[a]	16.06 ± 1.8	0.81 ± 0.19	63.92 ± 1.13	11.96 ± 0.68[a]	23.38 ± 0.85	1.26 ± 0.23	76.1 ± 2.01[a]	9.92 ± 1.02[a]	9.45 ± 2.1[a]

Values represent the percentage of the average ± SEM of three independent experiments. [a] indicates statistically significant differences with respect to control ($p < 0.05$)

Table 2 Percentage of live, apoptotic and dead cells after 24 h after exposure to PINO (0.001, 0.01, 0.1 1 10 and 100 μM)

	MDA-MB-231			MCF7			MCF10A		
	Live	Apoptotic	Death	Live	Apoptotic	Death	Live	Apoptotic	Death
Control	83.22 ± 5.33	14.82 ± 4.76	1.95 ± 0.6	92,03 ± 2,69	6,97 ± 2,9	1,26 ± 0,61	98.14 ± 0.47	1.33 ± 0.42	0.5 ± 0.08
0.001 μM	82.17 ± 4.76	15.56 ± 5.56	2.25 ± 0.94	92,38 ± 1,82	6,2 ± 1,83	1,07 ± 0,62	96.17 ± 0.76	2.73 ± 0.48	1.08 ± 0.29
0.01 μM	80.94 ± 8.15	16.24 ± 6.77	2.8 ± 1.57	93,14 ± 2,06	5,98 ± 1,69	0,85 ± 0,42	95.7 ± 1.44	3.45 ± 1.63	0.83 ± 0.37
0.1 μM	76.84 ± 9.32	20.15 ± 8.06	2.99 ± 1.34	92,47 ± 1,33	6,49 ± 1,08	1,03 ± 0,39	95.11 ± 2.6	3.99 ± 2.56	0.88 ± 0.16
1 μM	78.82 ± 7.41	18.05 ± 6.27	3.16 ± 1.29	93,32 ± 1,98	5,86 ± 1,61	0,8 ± 0,43	93.32 ± 1.51	5.85 ± 1.76	0.8 ± 0.25
10 μM	79.33 ± 7.24	17.75 ± 6.15	2.9 ± 1.19	93,89 ± 1,29	5,4 ± 1,08	0,69 ± 0,28	95.07 ± 1.1	4.17 ± 1.3	0.74 ± 0.2
100 μM	78.1 ± 6.84	18.46 ± 5.92	3.42 ± 1.09	91,65 ± 1,25	7,48 ± 1,14	0,85 ± 0,18	91.82 ± 2.33[a]	7.26 ± 2.54[a]	0.9 ± 0.23

Values represent the average ± SEM of three independent experiments. [a] was considered statistically significant respect to the control ($p < 0.05$)

components of its phenolic fraction, has been correlated with a low occurrence of breast cancer [1]. In fact, in the phenolic fraction of VOOs there are several compounds with anti-breast cancer properties as oleouropein [33], hydroxytyrosol and tyrosol [6]. Certain compounds showed more effectiveness in ER- breast cancer cells than in ER+ breast cancer cells [33]. PINO and 1-acetoxypinoresinol were first detected in VOO by Owen et al. [8] and differ in their relative amounts according to the different olives varieties used to make the VOO [9]. For example, Brenes et al. [10] reported that Spanish olive oil contains a range of 20 to 45 mg/kg PINO. Despite the well-established preventative role of phytoestrogens against breast cancer, very little research has been done to elucidate whether PINO plays a chemopreventive role or exhibits antitumor activity in human breast cancer cells. Moreover, the oestrogen receptor status is a key factor to consider in breast cancer therapy. In fact, hormone therapy is only used in oestrogen receptor-positive breast cancer [17, 18]. Accordingly, we attempted to elucidate the effects of PINO on human mammary cells with different oestrogen and progesterone receptor expression, to determine whether this compound may contribute, at least in part, to the reduced incidence of breast cancer associated with VOO consumption. For this purpose, we used the following human breast tumour cells: MDA-MB-231 cells (ER-, PR-) and MCF7 cells (ER+, PR +). Furthermore, non-tumorigenic human mammary epithelial cells were also used in the present study [MCF10A (ER-, PR-)].

Our results, summarized in Table 3, indicate that PINO showed cytotoxic, anti-proliferative and pro-oxidant activity in human breast tumour cells, independent of their oestrogen receptor expression levels. In addition, based on its effect in human mammary epithelial cells (Table 3), PINO may have chemopreventive activity, as induced antioxidant activity and prevented DNA from oxidative damage at a concentration of 1 μM. Interestingly, we found that PINO exerted differential activity on human breast tumour cells compared with mammary epithelial cells. Indeed, PINO treatment induced antioxidant activity in mammary epithelial cells, while it acted as a pro-oxidant molecule in human breast cancer cells after inducing oxidative stress.

The cytotoxic activity of PINO on human breast tumour cells is a debated issue. Previously, Chin et al. [25] described that PINO has a cytotoxic effect against MCF7 breast cancer cells (ED_{50} = 4.74 μM); however, in a later article [21], the same author found no cytotoxic effects. Surprisingly, the range of concentrations used in both studies was not specified. In addition, the cytotoxic effects of PINO in MDA-MB-231 cells have not been previously reported. In contrast, we tested a wide range of PINO concentrations and showed that there was cytotoxic activity at different concentrations in both human breast tumour cells tested. While PINO showed cytotoxic activity in both types of human breast tumour cells tested, the effect was more pronounced in negative oestrogen receptor tumour cells compared to oestrogen receptor-positive tumour cells (Figs. 4 and 5). In addition, for the first time, we describe the effects of PINO on human mammary epithelial cells. Our results suggest that PINO ranging between 0.001 and 0.1 μM, which could be considered as physiological doses, has a much greater cytotoxic effect on breast tumour cells compared to mammary epithelial cells, suggesting an anti-tumour effect of this compound with a minor damage to non-tumorigenic tissue.

Little research has been performed to understand the effects of PINO on human breast cancer cell proliferation. Sepporta et al. [27] found that PINO inhibited the growth of MDA-MB-231, but not of MCF7 cells; however, their study was limited to 100 μM, which is not considered to be a physiological concentration. In contrast, we tested a wide range of PINO concentrations, ranging from 0.001 to 100 μM, and showed that low concentrations of PINO elicited a significant antiproliferative effect on both human breast tumour cell lines tested. Future work is needed to clarify the mechanisms of inhibition of breast cancer cells growth only at low doses.

Oestrogen has been associated with the promotion and growth of breast cancer. In line with this result, most human breast cancers that are oestrogen-dependent undergo

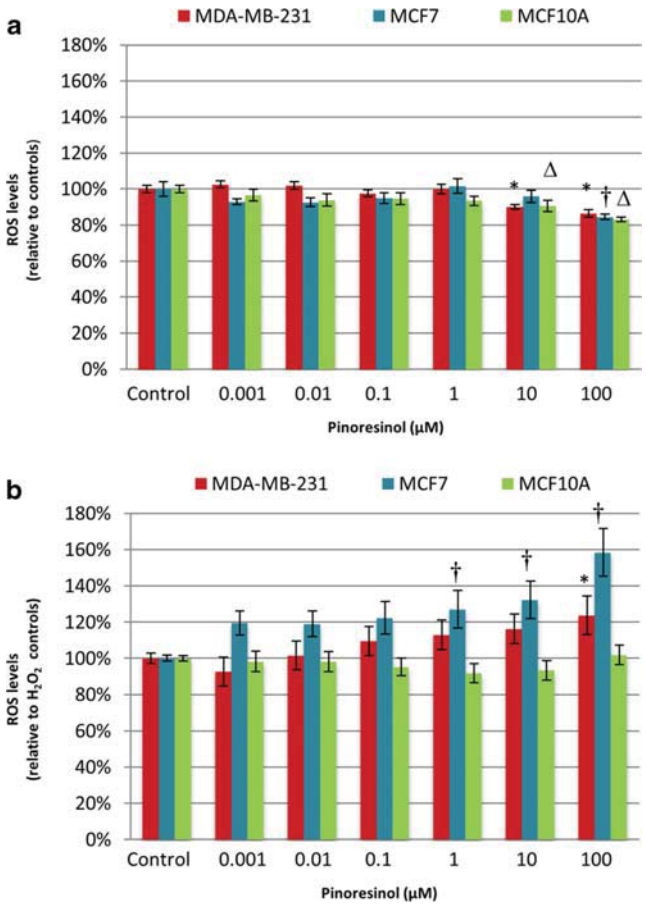

Fig. 6 DCFH-DA assay. Intracellular Reactive Oxygen Species (ROS) in breast cells treated with (+)-pinoresinol (in a range from 0.001 – 100 μM) for 24 h under basal conditions **a** and after H_2O_2-induced oxidative stress **b**. Data are presented as the mean ± SEM of three independent experiments and *(for MDA-MB-231), † (for MCF7) and Δ (for MCF10A) represent statistically significant differences ($p < 0.05$) with respect to the control, which was set as 100 %

regression when deprived of the supporting hormone [17]. Our results, therefore, are very interesting because although PINO is a phytoestrogen with an oestrogen-like chemical structure, it produced a decrease in the proliferation of human breast tumour cells. Thus, PINO could have oestrogen antagonist activity, like tamoxifen, which inhibits breast cancer cells proliferation. However, in the experimental cell model we designed, we used cell culture media without oestrogen supplementation, suggesting that PINO is not likely to act as an oestrogen antagonist. Interestingly, a previous prospective study showed that high dietary intakes of plant lignans, such as PINO, were associated with reduced risks of ER+/PR+ postmenopausal breast cancer [20]. We do not believe that the anti-proliferative effects of PINO are mediated by interactions with ERα because this receptor is not expressed in MDA-MB-231 breast tumour cells. Furthermore, the cell proliferation reduction was higher in MDA-MB-231 cells than in ERα + MCF7 breast cancer cells. On the other hand, it is unlikely that the anti-proliferative effects of PINO could be due to the activation of ERβ because both breast cancer cells tested MDA-MB-231 and MCF7, express low levels of this receptor [34]. Additionally, it has been suggested that ERβ exerts anti-proliferative effects in breast cancer cells in the presence of ERα, but exerts proliferative effects in the absence of ERα [17]. If this were true, treatment with PINO would result in an increase of MDA-MB-231 breast cancer cell (ERβ low/ ERα negative) proliferation. Instead, we found an anti-proliferative effect, which was even greater than that observed in MCF7 breast tumour

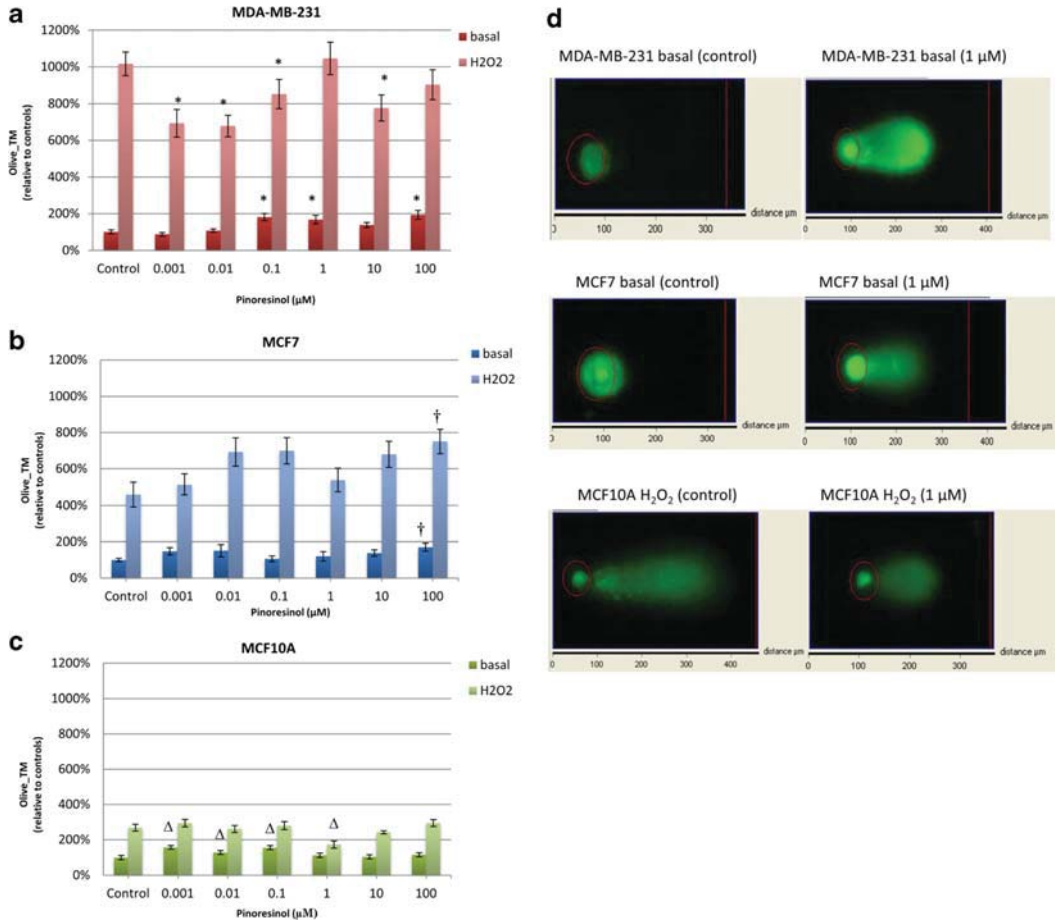

Fig. 7 Comet assay. DNA oxidative damage in MDA-MB-231 **a** MCF7 **b** or MCF10A **c** after (+)-pinoresinol treatments expressed as Olive_TM (mean ± SEM). The comet assay was performed under basal and H_2O_2-induced injury conditions. Data are expressed as the percentage relative to the basal (untreated) control, which was set as 100 %. Statistically significant differences (*, † or Δ) were established relative to basal or H_2O_2-treated control ($p < 0.05$). **d** Representative comet assay images showing different treatments on MDA-MB-231, MCF7 and MCF10A cells

cells (ERβ low/ ERα positive). Based on these results, we hypothesize that the anti-proliferative effects of PINO in the breast cancer cells assayed are independent of both ERα and ERβ status.

Previously, it has been shown that persistent ROS induction in non-tumorigenic cells may lead to cancer initiation, progression and spreading via activation and maintenance of signalling pathways that regulate cellular proliferation, survival, angiogenesis and metastasis [35]. However, we have not found previously published results regarding the antioxidant capacity of PINO in mammary cells. Our results suggest that PINO may prevent cancer development, as it diminished ROS levels in MCF10A mammary epithelial cells.

On the other hand, it is known that cancer cells possess higher intracellular ROS levels than non-tumorigenic cells and that enhanced ROS levels may be exploited to promote cancer cell death [36]. In fact, many of the commonly used chemotherapies are based on increasing oxidative stress above a toxic threshold level to selectively kill cancer cells [36]. In line with this concept, PINO may be used as a potential effective adjuvant to cancer therapies, as it was found to promote ROS generation in breast cancer cells, while it tended to diminish ROS induction in mammary epithelial cells. MCF7 cells were shown to be particularly sensitive to increased ROS levels after H_2O_2-induced oxidative stress, which could be related with the levels of DNA damage observed under basal conditions and after oxidative

Table 3 Summarized effects of PINO treatment on tumorigenic and non-tumorigenic mammary cells. a) Human breast tumour cells (MDA-MB-231 and MCF7) and b) non-tumorigenic human mammary epithelial cells (MCF10A)

a		Cytotoxicity	Proliferation	Oxidation (after H_2O_2 addition)	DNA damage (basal state)	DNA damage (after H_2O_2 addition)
MDA-MB-231 (ER-)	Concentrations (μM) with statistically significant differences	0.001 - 1	0.001 – 0.1	100	0.1, 1, 100	0.001 – 0.1, 10
MCF7 (ER+)	Concentrations (μM) with statistically significant differences	0.001 – 0.01	0.001 – 0.1	1 - 100	100	100
b		**Cytotoxicity**	**Proliferation**	**Oxidation (basal state)**	**DNA damage (after H_2O_2 addition)**	
MCF10A (ER-)	Concentrations (μM) with statistically significant differences	0.001, 10	0.01 - 10	10, 100	1	

shock. Under basal conditions, PINO also caused DNA damage in MDA-MB-231 cells; however, in contrast, PINO treatment prevented DNA damage in non-tumorigenic mammary epithelial cells, suggesting that PINO treatment may protect DNA in a pro-tumorigenic environment, thereby inhibiting breast cancer initiation and progression. Surprisingly, ER negative cells showed reduced DNA damage in response to H_2O_2, whereas ER positive cells showed an increase in DNA damage.

Very few reports have studied the chemical antioxidant capacity of PINO, and the results have varied considerably. For example, Kuo et al. [22] obtained a significant DPPH free radical scavenging activity for PINO, but these results differ from the work done by Chin et al. [21] and Vuorela et al. [23], which demonstrated a much higher IC_{50}. Our results suggest that PINO harbours a radical scavenging activity at concentrations of 10 μM or above for ABTS. This capacity was also shown using the DPPH method and is line with work published by Chin et al. [21]. In the ORAC assay, which is considered to be the most biologically relevant assay [24], PINO also showed antioxidant activity in a dose dependent manner.

Conclusions

Here, we showed that PINO possesses a chemical antioxidant capacity and may have a therapeutic potential to prevent breast cancer development via the reduction of intracellular oxidative stress and DNA damage in human mammary epithelial cells. Furthermore, we showed that PINO promotes an increase in the ROS levels of breast cancer cells after H_2O_2 treatment. In sum, this work suggests that PINO may act as adjuvant to pro-oxidative chemotherapies.

Finally, we showed that PINO has anti-tumour effects at low concentrations by promoting cytotoxic, anti-proliferative and pro-oxidant activities in breast cancer cells, independent of their oestrogen receptor status.

Additional files

Additional file 1: Representative flow cytometry analysis of the cell cycle of MDA-MB-231, MCF7 and MCF10A cells after treatment with (+)-pinoresinol. (DOCX 205 kb)

Additional file 2: Representative images of apoptosis analysis by flow cytometry in MDA-MB-231, MCF7 and MCF10A cells. (DOCX 567 kb)

Abbreviations

ABTS, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DCFH-DA, dichlorofluorescin diacetate; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ER, oestrogen receptor; FBS, foetal bovine serum; HER2, human epidermal growth factor receptor 2; NEAA, non-essential amino acids; ORAC, oxygen radical absorbance capacity; PINO, (+)-pinoresinol; PR, progesterone receptor; ROS, reactive oxygen species; RSA, radical scavenging activity; VOO, virgin olive oil.

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Availability of data and materials

The relevant datasets supporting the conclusions of this article are included within the article. The whole data including all images obtained, histograms,

analyses or raw data are available from the corresponding author on reasonable request.

Authors' contribution
Conception and design: JJG, AL-B, CS-Q; Development of methodology: AL-B, CS-Q; Sample processing: AL-B, CS-Q; Analysis of data: AL-B, JJG, MD-R; Writing of the manuscript: AL-B, JJG; Revision of the manuscript: JJG, GB, MD-R. All authors read and approved the final manuscript.

Competing interest
The authors declare that they have no competing interest.

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References

1. Toledo E, Salas-Salvado J, Donat-Vargas C, Buil-Cosiales P, Estruch R, Ros E, et al. Mediterranean diet and invasive breast cancer risk among women at high cardiovascular risk in the PREDIMED Trial: a randomized clinical trial. JAMA Intern Med. 2015;175(11):1752–60.
2. Sanchez-Quesada C, Lopez-Biedma A, Warleta F, Campos M, Beltran G, Gaforio JJ. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of Olea europaea. J Agric Food Chem. 2013;61(50):12173–82.
3. Warleta F, Campos M, Allouche Y, Sanchez-Quesada C, Ruiz-Mora J, Beltran G, et al. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Food Chem Toxicol. 2010;48(4):1092–100.
4. Allouche Y, Warleta F, Campos M, Sanchez-Quesada C, Uceda M, Beltran G, et al. 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. 2011;59(1):121–30.
5. Sanchez-Quesada C, Lopez-Biedma A, Gaforio JJ. Maslinic Acid enhances signals for the recruitment of macrophages and their differentiation to m1 state. Evid Based Complement Alternat Med. 2015;2015:654721.
6. Warleta F, Quesada CS, Campos M, Allouche Y, Beltran G, Gaforio JJ. Hydroxytyrosol protects against oxidative DNA damage in human breast cells. Nutrients. 2011;3(10):839–57.
7. Martin-Pelaez S, Covas MI, Fito M, Kusar A, Pravst I. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol Nutr Food Res. 2013;57(5):760–71.
8. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Identification of lignans as major components in the phenolic fraction of olive oil. Clin Chem. 2000;46(7):976–88.
9. Allouche Y, Jimenez A, Gaforio JJ, Uceda M, Beltran G. How heating affects extra virgin olive oil quality indexes and chemical composition. J Agric Food Chem. 2007;55(23):9646–54.
10. Brenes M, Garcia A, Garcia P, Rios JJ, Garrido A. Phenolic compounds in Spanish olive oils. J Agric Food Chem. 1999;47(9):3535–40.
11. Kulik T, Busko M, Pszczolkowska A, Perkowski J, Okorski A. Plant lignans inhibit growth and trichothecene biosynthesis in Fusarium graminearum. Lett Appl Microbiol. 2014;59(1):99–107.
12. Yang CP, Huang GJ, Huang HC, Chen YC, Chang CI, Wang SY, et al. The Effect of the Aerial Part of Lindera akoensis on Lipopolysaccharides (LPS)-Induced Nitric Oxide Production in RAW264.7 Cells. Int J Mol Sci. 2013;14(5):9168–81.
13. During A, Debouche C, Raas T, Larondelle Y. Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal Caco-2 cells. J Nutr. 2012;142(10):1798–805.
14. Wikul A, Damsud T, Kataoka K, Phuwapraisirisan P. (+)-Pinoresinol is a putative hypoglycemic agent in defatted sesame (Sesamum indicum) seeds though inhibiting alpha-glucosidase. Bioorg Med Chem Lett. 2012;22(16):5215–7.
15. Fini L, Hotchkiss E, Fogliano V, Graziani G, Romano M, De Vol EB, et al. Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. Carcinogenesis. 2008;29(1):139–46.
16. Hashim YZ, Rowland IR, McGlynn H, Servili M, Selvaggini R, Taticchi A, et al. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. Int J Cancer. 2008;122(3):495–500.
17. Haldosen LA, Zhao C, Dahlman-Wright K. Estrogen receptor beta in breast cancer. Mol Cell Endocrinol. 2014;382(1):665–72.
18. Ososki AL, Kennelly EJ. Phytoestrogens: a review of the present state of research. Phytother Res. 2003;17(8):845–69.
19. Leygue E, Murphy LC. A bi-faceted role of estrogen receptor beta in breast cancer. Endocr Relat Cancer. 2013;20(3):R127–39.
20. Touillaud MS, Thiebaut AC, Fournier A, Niravong M, Boutron-Ruault MC, Clavel-Chapelon F. Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. J Natl Cancer Inst. 2007;99(6):475–86.
21. Chin YW, Chai HB, Keller WJ, Kinghorn AD. Lignans and other constituents of the fruits of Euterpe oleracea (Acai) with antioxidant and cytoprotective activities. J Agric Food Chem. 2008;56(17):7759–64.
22. Kuo PC, Lin MC, Chen GF, Yiu TJ, Tzen JT. Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. J Agric Food Chem. 2011;59(7):3214–9.
23. Vuorela S, Kreander K, Karonen M, Nieminen R, Hamalainen M, Galkin A, et al. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. J Agric Food Chem. 2005;53(15):5922–31.
24. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005;53(10):4290–302.
25. Chin YW, Jones WP, Rachman I, Riswan S, Kardono LB, Chai HB, et al. Cytotoxic lignans from the stems of Helicteres hirsuta collected in Indonesia. Phytother Res. 2006;20(1):62–5.
26. Menendez JA, Vazquez-Martin A, Oliveras-Ferraros C, Garcia-Villalba R, Carrasco-Pancorbo A, Fernandez-Gutierrez A, et al. Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med. 2008;22(4):433–9.
27. Sepporta MV, Mazza T, Morozzi G, Fabiani R. Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. Nutr Cancer. 2013;65(8):1208–18.
28. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9–10):1231–7.
29. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol. 1995;28:25–30.
30. Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. J Agric Food Chem. 2003;51(11):3273–9.
31. Sanchez-Quesada C, Lopez-Biedma A, Gaforio JJ. The differential localization of a methyl group confers a different anti-breast cancer activity to two triterpenes present in olives. Food Funct. 2015;6(1):249–56.
32. Lee DH, Lim BS, Lee YK, Yang HC. Effects of hydrogen peroxide (H2O2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. Cell Biol Toxicol. 2006;22(1):39–46.
33. Elamin MH, Daghestani MH, Omer SA, Elobeid MA, Virk P, Al-Olayan EM, et al. Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. Food Chem Toxicol. 2013;53:310–6.
34. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep. 2000;7(1):157–67.
35. Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. Biochem Pharmacol. 2014;92(1):90–101.
36. Nogueira V, Hay N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. Clin Cancer Res. 2013;19(16):4309–14.

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The biological activities of natural lignans from olives and virgin olive oils: A review

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ABSTRACT

Dietary guidelines recommend diets rich in plant foods, because they are associated with a lower incidence of chronic diseases, such as cardiovascular disease and certain cancers. The compounds that exhibit these health benefits are a matter of debate; however, scientific evidence assigns an important role to the action of so-called minor compounds. Lignans are polyphenols found in plants, and they are part of the phytoestrogen family, which is known for its health properties. The natural lignans (+)-pinoresinol and 1-acetoxypinoresinol are typically found in olives and, consequently, virgin olive oils (VOOs), which are genuine fruit juices. Although (+)-pinoresinol has been identified in other plants, 1-acetoxypinoresinol is specifically observed in olives. In this review, we collected information regarding these two main lignans found in VOOs, because a number of researchers believe that they may play a prominent role in the health effects attributed to virgin olive oils.

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1. Introduction

The incidence rates of certain chronic diseases, such as certain cancers and cardiovascular disease, are higher in Western countries, where people consume a diet rich in fat and animal protein, compared to countries that follow a Mediterranean diet rich in plant foods that contain phytoestrogens. Therefore, it has been postulated that phytoestrogens may be involved in conveying the beneficial effects attributed to the Mediterranean diet. In fact, phytoestrogens are abundant in the plasma and urine of subjects living in areas with a low cancer incidence rate (Adlercreutz, 2007).

The two main groups of phytoestrogens are isoflavonoids and lignans. It is widely assumed that the intake of lignan-rich foods is part of a healthy diet, and several reviews have conveyed information about lignans derived from different sources in the plant kingdom, as flaxseeds and sesame (Dar & Arumugam, 2013; Kajla, Sharma, & Sood, 2015; Pan et al., 2009; Saleem, Kim, Ali, & Lee, 2005; Umezawa, 2003). However, this is the first review that describes, specifically, the lignans typically found in olives and virgin olive oils (VOOs) and their implications on health and several diseases. These natural lignans found in olives and VOOs, which is the main source of fat in the Mediterranean diet, are (+)-pinoresinol and 1-acetoxypinoresinol. In fact, although (+)-pinoresinol has been identified in other plants, 1-acetoxypinoresinol is specifically found in olives. It is widely accepted that the consumption of lignans (Adlercreutz, 2007; Lin, Yngve, Lagergren, & Lu, 2012; Peterson et al., 2010) and VOOs (Toledo et al., 2015), provide health benefits, and several studies have highlighted beneficial effects of (+)-pinoresinol and 1-acetoxypinoresinol characteristics of VOOs (Menendez et al., 2008a; Owen et al., 2000a). Consequently, both (+)-pinoresinol and 1-acetoxypinoresinol represent a high interest based on their biological and pharmacological properties. Unfortunately, the information known about these compounds is largely ambiguous and diverse, making more work for researchers who want to study and gather information on these compounds. Thus, this review summarizes the current published information related to the bioactivity and potential health benefits associated with (+)-pinoresinol and 1-acetoxypinoresinol, as well as their chemical characteristics, distribution and metabolism in humans. In addition, this study highlights the importance of further studying these compounds, due to their pharmacological potential.

2. Classification, chemical structure and metabolic pathway

Polyphenols are secondary metabolites in plants that are involved in the defence against ultraviolet radiation and aggressive pathogens (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). This group includes the lignans pinoresinol and 1-acetoxypinoresinol, the subjects of this review.

Specifically, pinoresinol ($C_{20}H_{22}O_6$) and 1-acetoxypinoresinol ($C_{22}H_{24}O_8$) are phenolic compounds that form a dimer, which means they possess two phenol groups in their chemical structure (Fig. 1). Each phenol group consists of an aromatic ring (phenyl or benzene group) bound to a hydroxyl group (OH). Both, the phenol group and the benzene ring are associated with several health benefits in humans including antioxidant and/or anti-inflammatory effects (During, Debouche, Raas, & Larondelle, 2012; Sok, Cui, & Kim, 2009; Yang et al., 2013).

The molecular weights of pinoresinol and 1-acetoxypinoresinol are 358.38 and 416.42 g/mol, respectively. Their structures are very similar, except for the appearance of a -$COOCH_3$ group in 1-acetoxypinoresinol that is not present in pinoresinol.

The biosynthesis of pinoresinol has been investigated in *Forsythia* spp (Kim et al., 2009; Umezawa, 2003). This compound is synthesized by the stereospecific coupling of two units of

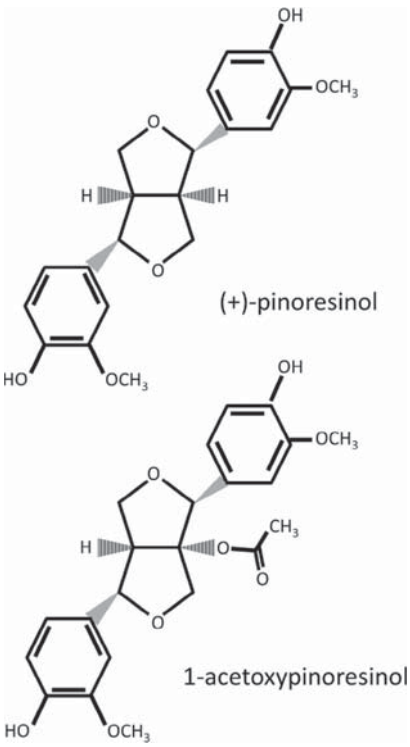

Fig. 1 – Chemical structures of (+)-pinoresinol and 1-acetoxypinoresinol.

coniferyl alcohol in the presence of a dirigent protein (DIR). It is then metabolized to lariciresinol and secoisolariciresinol via pinoresinol-lariciresinol reductase (PLR) or to piperitol and sesamin via piperitol/sesamin synthase (PSS). No references have reported the biosynthesis of 1-acetoxypinoresinol.

3. Distribution

Lignans are distributed widely in the plant kingdom, with flaxseeds being a major source (Thompson, Chen, Li, Strasser-Weippl, & Goss, 2005). Despite this fact, 1-acetoxypinoresinol has not been identified in flaxseeds and pinoresinol is present at very low concentrations in comparison to other lignans. It has been reported that the contribution of flaxseeds to the mean intake of pinoresinol is only 0.3% (Milder et al., 2005). 1-acetoxypinoresinol has been found only in the bark of the olive tree (*Olea europaea*) (Tsukamoto, Hisada, & Nishibe, 1984), while pinoresinol has been found in several species including *Forsythia* spp., *Linum flavum, Brassica* spp., *Daphne* spp., *Larix leptolepis*, *Sesamun indicum, Pinus* spp., *Picea jezoensis, Tsuga heterophylla, Araucaria angustifolia, Fraxinus mandshurica, Wikstroemia* spp., *Osmanthus fragans*, *Trichosanthes kirilowii*, *Sophora tonkinensis*, *Piper wallichii*, the stems of *Viburnum erosum*, the seeds of *Melia azedarach* and the roots of *Rosa multiflora*, among others (Carpinella, Giorda, Ferrayoli, & Palacios, 2003; Harborne, Baxter, & Moss, 1999; In et al., 2015; Lee et al., 2015; Liu et al., 2015; Minh et al., 2015; Owen et al., 2000b; Shi et al., 2015; Sok et al., 2009; Umezawa, 2003; Yeo, Chin, Park, & Kim, 2004).

In the year 2000, two independent studies (Brenes et al., 2000; Owen et al., 2000a) have identified, for the first time, pinoresinol and 1-acetoxypinoresinol in VOO; it was the enantiomer form of (+)-pinoresinol that was identified. This finding has attracted special interest, because the consumption of VOO is associated with beneficial properties that could result from the presence of these lignans as predominant compounds in its phenolic fraction. In fact, Owen et al. (2000a, 2000b) noted that these compounds are not present (or virtually absent) in seed oils and refined oils, but they are major components of the phenolic fraction of virgin olive oils, with concentrations up to 100 mg/kg. These researchers also found considerable variation in the concentrations of these compounds within different oils, but the average total lignan concentration was 41.53 ± 3.93 mg/kg. Similarly, Brenes, Garcia, Dobarganes, Velasco, and Romero (2002a) found differences among 10 extra virgin olive oils, with concentrations varying from 11.7 to 41.2 mg/kg for (+)-pinoresinol and 2.7 to 66.9 mg/kg for 1-acetoxypinoresinol.

Since then, several studies have addressed the quantification of total phenolic or lignan content in VOO, as well as the concentrations of individual phenolic compounds. However, it is difficult to provide concrete data due to the high number of variables that can affect the concentrations of these compounds. These variables include olive variety, cultivation area, climate, harvesting, degree of ripening, stoning, production techniques, transport, storage and experimental procedures for isolation and analysis.

Time, temperature and oxygen concentration during malaxation (beating of the paste) also affect the phenolic concentrations (Servili et al., 2007), although some researchers did not find significant changes in the concentration of (+)-pinoresinol or 1-acetoxypinoresinol under different conditions of malaxation (García et al., 2001). In addition, Gambacorta et al. (2010) reported that the degree of maturation did not have a significant influence on the concentration of 1-acetoxypinoresinol, as Bonoli, Bendini, Cerretani, Lercker, and Toschi (2004) noted for both compounds. However, these researchers reported differences related to the technology used to obtain the VOO, because oils from stoned olives showed higher concentrations of 1-acetoxypinoresinol, which was also reported by Servili et al. (2007); the latter case, the variations were not significantly different. Gambacorta et al. (2010) also noted that 1-acetoxypinoresinol concentrations were influenced by storage time, resulting in a slight increase after 6 months of storage and a significant decrease after 12 months of storage.

A previous study (Kalua, Bedgood, Bishop, & Prenzler, 2008) reported that levels of pinoresinol diminished gradually during 4 weeks of storage, while 1-acetoxypinoresinol levels were enhanced by weeks 1 and 3. In contrast, Brenes, Garcia, Garcia, and Garrido (2001) found that concentrations of pinoresinol and 1-acetoxypinoresinol did not change during a one-year storage period under commercial conditions (darkness and lack of oxygen). These researchers also observed minor losses of both compounds when VOO was heated, which is an important aspect considering that VOO is not only consumed raw but is also heated during frying, boiling or microwave heating (Brenes et al., 2002a).

In short, pinoresinol and 1-acetoxypinoresinol appear more stable than other phenolic compounds under different conditions of malaxation, olive stoning, maturity or storage time (Bonoli et al., 2004; Brenes et al., 2001; García et al., 2001; Servili et al., 2007). However, given the high number of variables involved in obtaining the final oil product, the concentrations of these lignans in VOOs differ widely.

Despite variations in lignan concentrations, lignans exist in certain proportions in different oil varieties. For example, 1-acetoxypinoresinol is very abundant in Arbequina, Empeltre and Hojiblanca oils but is present in very low concentrations in Picual oils. Therefore, this compound has been proposed as a potential indicator of authenticity for Picual oils (Brenes, García, Rios, García, & Garrido, 2002b; Gómez Caravaca, Carrasco Pancorbo, Canabate Diaz, Segura Carretero, & Fernandez Gutierrez, 2005). For guidance, Table 1 gives the concentrations found in different oil varieties.

The location of these compounds inside the olive tree remains unclear. Some researchers, such as Owen et al. (2000b), found that these lignans are not present in the pericarp of the olive drupes or in the leaves and twigs that are present in the mulch prepared for olive pressing. However, (+)-pinoresinol was found to be a major component of the phenolic fraction of olive piths. Furthermore, Bonoli et al. (2004) found pinoresinol and a small amount of 1-acetoxypinoresinol in the bark of olive fruit trees from Italian cultivars, but none of the compounds was detected in the extracts of seeds and pulp. Similar results were obtained by Oliveras López et al. (2008) who found pinoresinol in the olive stones of four olive varieties. They did not find pinoresinol or 1-acetoxypinoresinol in the pulp, although a small amount of 1-acetoxypinoresinol was found in the Taggiasca

Table 1 – Concentrations (mg/kg) of (+)-pinoresinol and 1-acetoxypinoresinol found in VOO obtained from different olive varieties. U = unspecified.

Olive variety	(+)-pinoresinol (mg/kg)	1-acetoxypinoresinol (mg/kg)	Reference
Arbequina	41.2–34	66.9 - 36.4	(Brenes et al., 2000)
	9.99 ± 0.32	36.2 ± 1.45	(Gómez Caravaca et al., 2005)
	2.83 ± 0.08	5.12 ± 0.95	(Chtourou, Gargouri, Jaber, Abdelhedi, & Bouaziz, 2013)
	46.2	77.3	(García et al., 2001)
Brisighella	12.35 ± 0.9	23.4 ± 1.2	(Antonini et al., 2015)
Chemlali Sfax	1.70 ± 0.03	6.23 ± 0.5	(Chtourou et al., 2013)
Coratina	U	11.32 - 6.88	(Gambacorta et al., 2010)
	8.4 ± 0.6	41.1 ± 1.1	(Servili et al., 2007)
Cornicabra	4.37 ± 1.33	1.2 ± 0.045	(Gómez Caravaca et al., 2005)
	74.5 ± 0.4	4.9 ± 0.2	(Selvaggini et al., 2006)
Empeltre	19 - 11.7	40 - 31.5	(Brenes et al., 2000)
Frantoio	55.1 ± 0.1	58.1 ± 0.1	(Selvaggini et al., 2006)
	17 ± 8	82 ± 8	(Kalua et al., 2008)
	4.2 ± 0.4	40.7 ± 2.3	(Servili et al., 2007)
Hojiblanca	30.8 - 24.4	30.5 - 3.7	(Brenes et al., 2000)
	6.97 ± 0.25	3.42 ± 0.14	(Gómez Caravaca et al., 2005)
	53.2 ± 0.4	78.2 ± 1.7	(Selvaggini et al., 2006)
Lechín de Granada	3.48 ± 0.13	0.86 ± 0.03	(Gómez Caravaca et al., 2005)
Lechín de Sevilla	0.62 ± 0.02	26.78 ± 0.99	(Gómez Caravaca et al., 2005)
Picual	36 - 29.5	4.9 - 2.7	(Brenes et al., 2000)
	6.94 ± 0.28	0.167 ± 0.006	(Gómez Caravaca et al., 2005)
	33.8	U	(García et al., 2001)
Picudo	31.2 - 29.1	12.5 - 6.8	(Brenes et al., 2000)
	4.49 ± 0.16	0.58 ± 0.02	(Gómez Caravaca et al., 2005)
Riviera ligure	8.8 ± 0.01	27.1 ± 1.15	(Antonini et al., 2015)
Sariulak	45.3–109.3	54.3–123.5	(Arslan, Karabekir, & Schreiner, 2013)
Tuscia	8.9 ± 0.1	15.7 ± 1.7	(Antonini et al., 2015)

variety. Servili et al. (2007) detected lignans in both the pulp and the stone but not in the seeds of olive fruits. 1-acetoxypinoresinol was the more prevalent lignan in the pulp, and pinoresinol was the more prevalent lignan in the stone. By contrast, Kalua et al. (2008) detected these lignans in olive oil but not in the olive fruits of the Frantoio variety.

It is worth noting that although these lignans are sometimes present in VOOs, they are not found in the fruits from which the oils originate. One plausible explanation could be that the techniques used for extraction in these studies failed to detect the compounds. For instance, Oliveras López et al. (2008) tried several methods to extract pinoresinol and 1-acetoxypinoresinol from stones and pulp of Italian and Spanish olive varieties known to produce VOOs rich in both lignans. Surprisingly, only one of the extraction methods was able to yield detectable levels of pinoresinol in the stones of the selected varieties. Another possible explanation could be that lignans, initially absent in olive fruits, are generated by the activity of β-glucosidase and the acidic conditions that arise when the olives are pasted during olive oil production (Soler et al., 2010). Finally, it is possible (though less likely) that lignans present in other parts of the olive tree that are included in the olive oil production process, such as stones, twigs and leaves, become incorporated in the final olive oil product.

4. Bioactivity

Lignans have been associated with several health properties such as protection against LDL oxidation and inhibition of cancerous cell growth in skin, breast, prostate, colon and lung tissues (Hirano et al., 1990; Kardono, Tsauri, Padmawinata, Pezzuto, & Kinghorn, 1990). Because lignans have chemical structures similar to oestrogen, they could act as hormonal modulators in breast cancers through oestrogenic or antiestrogenic activities. In fact, clinical trials have reported a reduction in the risk of postmenopausal breast cancer, specifically the ER^+/PR^+ subtype, in women with a high intake of plant lignans (Touillaud et al., 2007) and a reduction in breast tumour growth in patients who consume flaxseed daily (Thompson et al., 2005).

However, due to the broad range of compounds included in the lignan group, it would be advantageous to study the properties and biological activities of each one individually, because small variations in chemical structure may cause different or even opposite effects.

4.1. *Free radical scavenging activity*

Experimental data on 1-acetoxypinoresinol is fairly limited. This compound is not commercially available and difficulties in its isolation and synthesis have limited the study of its effects. Regarding its potential as a free radical scavenger, this compound elicited stronger responses than the standards Trolox and dimethyl sulphoxide in reactive oxygen species (ROS) attacks on salicylic acid in hypoxanthine/xanthine oxidase assays, where the inhibitory concentrations (IC_{50}) were 0.91, 12.24 and 2.30 mM for 1-acetoxypinoresinol, Trolox and dimethyl sulphoxide, respectively (Owen et al., 2000a). This same study also reported that 1-acetoxypinoresinol had a higher radical

Table 2 – Radical scavenging activity of (+)-pinoresinol and 1-acetoxypinoresinol.

Compound	Assay	IC_{50}	Reference
(+)-pinoresinol	DPPH	24.16 μM	(Kang & Wang, 2010)
		34.5 μM	(Kuo et al., 2011)
		96.82 μM	(Chin et al., 2008)
		558 μM	(Yi et al., 2011)
	ABTS	8.6 μM	(Kang & Wang, 2010)
		153.46 μM	(Yi et al., 2011)
		5 μM	(Koch et al., 2015)
1-acetoxypinoresinol	Hypoxanthine/xanthine oxidase	0.91 mM	(Owen et al., 2000a)

scavenging activity than the catechol derivatives caffeic acid, hydroxytyrosol and oleuropein.

Research using the DPPH method showed that both lignans were antioxidants, but (+)-pinoresinol had a greater effect than 1-acetoxypinoresinol (Carrasco-Pancorbo et al., 2005). However, these authors noted that both compounds had a pro-oxidant effect when used in a lipid model system (OSI), this effect being much higher for (+)-pinoresinol. As mentioned above, the chemical structure of this compound could justify the results of this study, because the $–COOCH_3$ group is not present in (+)-pinoresinol (Fig. 1). This group is not an electron donor, which may explain the lower antioxidant capacity of 1-acetoxypinoresinol in DPPH assays. Moreover, the pro-oxidant effect observed with the OSI method could be due to the oxygen atoms in each central ring contained in both molecules, which could cause an opening of the ring under the thermal conditions used in the assay. However, the $–COOCH_3$ group of 1-acetoxypinoresinol could hinder the opening of the ring, producing a weaker effect (Carrasco-Pancorbo et al., 2005).

Once again, the method of measurement seems to be the cause of conflicting results, but there are also differences among results derived from the same procedure. For example, with the DPPH assay, Kang and Wang (2010) observed high levels of radical scavenging activity for (+)-pinoresinol using similar concentrations of compounds to Kuo, Lin, Chen, Yiu, and Tzen (2011) (IC_{50} values were 24.16 μM by Kang & Wang, 2010, and 34.5 μM by Kuo et al., 2011). However, these values were much lower than those obtained by Chin, Chai, Keller, and Kinghorn (2008) (IC_{50} = 96.82 μM), Yi et al. (2011) (IC_{50} = 558 μM) and Vuorela et al. (2005) (percent of DPPH inhibition ± SD = 18.8 ± 1.3 for 1395 μM). Moreover, Li, Zhai, Tang, and Duan (2010) found no antioxidant activity for (+)-pinoresinol using this method. For ABTS testing, a recent study detected a noticeable radical scavenging activity (Koch, Buchter, Havermann, & Watjen, 2015). Kang and Wang (2010) and Yi et al. (2011) also observed significant antioxidant activity, but IC_{50} values varied considerably (8.6 μM (Kang & Wang, 2010) and 153.46 μM (Yi et al., 2011)).

Data related to the radical scavenging activities of (+)-pinoresinol and 1-acetoxypinoresinol are shown in Table 2.

4.2. *Biological activity* in vitro

In vitro studies showed that 1-acetoxypinoresinol completely reduced the fatty acid synthase (FASN) levels in HER2 gene-amplified SKBR3 breast cancer cells, which naturally overexpress this protein, and in MCF7 breast cancer cells engineered to overexpress the HER2 tyrosine kinase receptor (Menendez et al., 2008a). (+)-pinoresinol also diminished these levels, although to a lesser extent (30–35% reduction for SKBR3 cells and 50% for MCF7 cells). FASN protein levels are frequently altered in cancer cells, and this protein has been implicated in the activity and/or expression of key cancer-related oncogenes such as the Type I receptor tyrosine kinase HER2, whose overexpression enhances breast cancer cell proliferation, survival, chemoresistance and metastasis. Therefore, 1-acetoxypinoresinol and (+)-pinoresinol could be useful in the chemoprevention and/or treatment of breast carcinomas with FASN overexpression that result from oncogenic signalling driven by HER2 (Menendez et al., 2008b). The same authors reported significant cytotoxicity in SKBR3 cells, as well as reduced cell proliferation, apoptosis and inhibition of HER2 protein kinase activity, when VOO phenolic fractions were applied (Menendez et al., 2008a). The phenolic fraction, containing mainly 1-acetoxypinoresinol, was more effective than the fraction that contained mainly (+)-pinoresinol. Moreover, in MCF7/HER2 cells, the first phenolic fraction increased apoptosis greater than 4-fold and reduced the proliferation rate by 63% and HER2 expression by 83% (Menendez et al., 2008a). However, it is not known whether these effects were due to the presence of 1-acetoxypinoresinol or (+)-pinoresinol, because, the fractions contained small quantities of other polyphenolic compounds that could be contributing to the above mentioned activities.

Menendez et al. (2009) studied the effects of these lignans individually on breast epithelial cells (MCF10A) engineered to overexpress the wild-type form of human HER2. Remarkably, the compounds were able to down-regulate HER2 protein expression (≈30% reduction for (+)-pinoresinol and >70% for 1-acetoxypinoresinol), but further HER2 tyrosine kinase activity was decreased after the treatments, up to 70% in the case of 1-acetoxypinoresinol. The IC_{50} values required to decrease HER2 Y1248 phosphorylation were 64 μM of (+)-pinoresinol and 30 μM of 1-acetoxypinoresinol. Furthermore, the morphology of treated MCF10A/HER2 cells underwent striking changes including diminished growth density, expanded volume and the appearance of extrusions. Exposure to 1-acetoxypinoresinol inhibited the growth of cells and resulted in cell morphologies compatible with apoptosis. The authors suggested that the inhibitory effects were due to the presence of two or more phenol rings, because the single phenols and phenolic acids studied did not efficiently block HER2 tyrosine kinase activity (Menendez et al., 2009).

To date, few references have addressed the *in vitro* or *in vivo* activities of 1-acetoxypinoresinol. Studies have been carried

out for pinoresinol, most of which have reported activities including antifungal, anti-inflammatory, antioxidant or hypoglycaemic activities (During et al., 2012; Jung et al., 2010; Kulik, Busko, Pszczolkowska, Perkowski, & Okorski, 2014; Wikul, Damsud, Kataoka, & Phuwapraisirisan, 2012).

It is difficult to reach conclusions with respect to the possible cytotoxic effect of pinoresinol on cancer cells, because results vary broadly depending on the cell line used. Chin et al. (2006) observed that pinoresinol was cytotoxic in MCF7 (human breast cancer), LNCaP (hormone-dependent human prostate cancer) and Lu1 (human lung cancer) cells. In contrast, in a later article (Chin et al., 2008), the same authors found no cytotoxic effects in MCF7 cells, even observed cell cytoprotection when stressed the cells by H_2O_2. Previously, pinoresinol exhibited cytotoxicity in KB (HeLa derivative) cells with an IC_{50} = 2.2 μg/mL (Chiung et al., 1994), but no activity was observed in HL60 human promyelocytic leukaemia cells (Mimaki, Kuroda, Asano, & Sashida, 1999).

Fini et al. (2008) observed that pinoresinol inhibited cell viability and arrested the cell cycle in G2/M phase only in p53-proficient cells when tested in colon cancer cell lines with different expression levels of p53 gene. A synergistic effect together with other polyphenols present in the phenolic fraction of VOOs was suggested by these authors, because lower concentrations of pinoresinol were necessary to achieve cytotoxicity when using pinoresinol-rich extracts on human colon cancer cells. The use of these extracts decreased cell viability and induced apoptosis and G_2/M cell cycle arrest, presumably through the upregulation of ATM/p53 pathway. However, after using 100 μM of (+)-pinoresinol by itself, Sepporta, Mazza, Morozzi, and Fabiani (2013) noted that the antiproliferative activity was not always linked to the presence of functional p53. In human breast cancer cells, these researchers obtained this same effect in a cell line with a p53 mutation (MDA-MB-231) but not in p53-proficient cells (MCF7). In fact, they tested a panel of p53 proficient and deficient cell lines and, contrary to Mimaki et al. (1999), p53 deficient HL60 cells were the most sensitive to growth inhibition, with an IC_{50} = 8 μM.

The $p21^{WAF1/Cip1}$ protein, which is a modulator of biological functions associated with proliferation, apoptosis and differentiation, seems to play a role in the growth of cells treated with (+)-pinoresinol, which strongly induced its expression at both the protein and mRNA levels, resulting in antiproliferative effects (Sepporta et al., 2013). Cytotoxicity has also been reported in lung adenocarcinoma (A549), ovarian adenocarcinoma (OK-OV-3), skin melanoma (MEL-2) and colon carcinoma (HCT15) cells with IC_{50} values ranging from 26–34 μg/mL (Kwak, Kang, Roh, Choi, & Zee, 2009).

Despite the relevance of invasion to cancer progression and metastasis, there is only one study that covers the possible anti-invasive effects of pinoresinol. This study was carried out on human colon adenocarcinoma cells (HT115), in which pinoresinol showed significant anti-invasive effects when used at a range of concentrations from 1.56 to 25 μM, with a reduction of up to 65% when used at its lowest concentration (Hashim et al., 2008).

As an antifungal, pinoresinol was able to inhibit the growth of the pathogenic fungi *Fusarium verticilloides* (Carpinella et al., 2003) and *Fusarium graminearum*, as well as decreased trichothecene biosynthesis (Kulik et al., 2014). Hwang, Lee, Liu, Woo, and Lee (2010) also described pinoresinol's effectiveness as an antifungal, with minimum inhibitory concentrations (MIC) of 12.5 μg/mL for the human fungi *Candida albicans* and 25 μg/mL for both *Trichosporon beigelii* and *Malassezia furfur*. These values represent a less potent antifungal activity than the positive control Amphotericin B, which is a fungicidal agent used to treat several life-threatening infections in humans. Likewise, pinoresinol did not exhibit haemolytic activity against human erythrocytes, which is one of the side effects associated with Amphotericin B (Hwang et al., 2010).

However, (+)-pinoresinol did inhibit $CuSO_4$-induced peroxidation of low-density lipoprotein (LDL) in a concentration-dependent manner from 0.1 μM to 10 μM (Kang, Naito, Sakai, Uchida, & Osawa, 2000). The oxidation of LDL plays an important role in the formation of atherosclerotic plaques, and thus lignans could be used to reduce the appearance of the first signs of atherosclerosis in cardiovascular disease (Rafieian-Kopaei, Setorki, Doudi, Baradaran, & Nasri, 2014).

(+)-Pinoresinol was also effective as an antioxidant for liposomes, as well as an anti-inflammatory agent through its inhibition of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as observed in activated J774 murine macrophages (Vuorela et al., 2005). Jung et al. (2010) also reported pinoresinol anti-inflammatory activity in the primary microglia of rats, along with the inhibition of other proinflammatory mediators such as COX-2, TNFα, IL1β, IL-6 and NF-κB. During et al. (2012) observed a decrease in IL-6, PGE_2 and NF-κB levels after treatment in human colon adenocarcinoma cells (Caco-2). These authors associated the anti-inflammatory properties of pinoresinol with its furofuran structure, given that other lignans without this structure did not produce these effects. Several recent studies performed in LPS-activated RAW264.7 macrophages confirmed that NO production could be inhibited by pinoresinol, albeit with varying IC_{50} values (In et al., 2015; Lee et al., 2015; Liu et al., 2015; Yang et al., 2013). Therefore, (+)-pinoresinol could potentially be used to treat inflammatory diseases, specifically those associated with NO overproduction.

Furthermore, the addition of this lignan maintained cell viability and induced a protective effect against glutamate-induced cell death in mouse hippocampal HT22 cells, suggesting neuroprotective activity (In et al., 2015).

(+)-Pinoresinol has also been linked with hypoglycaemic agents because it inhibited rat intestinal α-glucosidase. This action is essential for the effective reduction of blood glucose levels and therefore could play an important role in treating metabolic disorders, such as type 2 diabetes mellitus (T2DM) (Wikul et al., 2012).

4.3. *Biological activity* in vivo

There are few studies that have investigated the *in vivo* effects of pinoresinol and those that have focused on very different areas. For example, in rats, this compound exhibited a dose-dependent protective effect against microvascular damage induced by hypoperfusion and reperfusion in pial circulation. Pinoresinol was able to induce vasodilation through reactive hyperaemia and reduce the generation of reactive oxygen species (ROS), protecting arteriolar wall integrity and capillary perfusion. Consequently, it also protected cerebral

blood flow distribution and prevented brain injury (Lapi et al., 2015).

In mice, pinoresinol protected against CCL_4-induced acute hepatotoxicity and attenuated the release of transaminases ALT and AST, which is a process indicative of severe hepatocellular damage. These effects were supported by histological observations of liver sections. Pinoresinol also decreased inflammatory responses through the reduction of TNF-α, iNOS and COX-2 protein and mRNA expression, as well as the transactivation of NF-κβ and AP-1 (Kim et al., 2010).

Pinoresinol was also tested in *Caenorhabditis elegans* by Koch et al. (2015). Although these researchers detected noticeable radical scavenging activity in the ABTS assay, they did not detect any antioxidant effects in the nematodes *in vivo*. However, pinoresinol induced nuclear translocation of DAF-16, a pivotal transcription factor of the insulin/IGF-like signalling pathway that regulates the expression of protective genes such as superoxide dismutase.

Finally, a case-control study showed that high-level consumption of pinoresinol is associated with a significant reduction in breast cancer risk among premenopausal women (Torres-Sanchez, Galvan-Portillo, Wolff, & Lopez-Carrillo, 2009). However, dietary habits during the 12-month period prior to the onset of symptoms were only assessed in patients and control subjects after breast cancer was diagnosed, and no daily follow-up to the study was pursued, posing a limitation in the real value of pinoresinol consumption. Thus, more clinical trials are needed to confirm the relationship between the intake of pinoresinol and a low breast cancer risk.

Table 3 summarizes the *in vitro* and *in vivo* bioactivities associated with (+)-pinoresinol and 1-acetoxypinoresinol.

5. Bioavailability, absorption and pharmacokinetics

Although we are aware of many of the effects exhibited by pinoresinol and 1-acetoxypinoresinol, we must also consider whether the concentrations used in testing are similar to those naturally found in the organism after consumption. Several factors, including bioavailability (the tendency of the compound to be extracted from the food matrix) (Soler et al., 2010), metabolism by colonic bacteria and absorption by intestinal or target cells, must be taken into account.

Little information has been published about these events with regards to 1-acetoxypinoresinol, although this compound has been shown to possess interesting biological activities.

5.1. *Intake*

The median intake of pinoresinol has been estimated for Dutch (Milder et al., 2005), French (Touillaud et al., 2007), Canadian (Cotterchio, Boucher, Kreiger, Mills, & Thompson, 2008) and Finnish (Nurmi, Mursu, Peñalvo, Poulsen, & Voutilainen, 2010) populations, with values of 312, 422, 107 and 442 μg/d, respectively. Plant lignans assessed in these studies were derived mainly from wholegrain and rye products, berries, coffee, tea, vegetables, and fruit. Nevertheless, the daily intake of pinoresinol is expected to be much higher in regions with a Mediterranean diet, where consumption of olives and VOOs is higher. In addition, olives and VOOs are the only edible sources of 1-acetoxypinoresinol and, consequently, the only foods that could provide its potential health benefits.

5.2. *Metabolism*

Pinoresinol and 1-acetoxypinoresinol are quite stable under gastric and duodenal digestion conditions, as proven *in vitro* with models simulating both conditions (Soler et al., 2010). In fact, the concentrations of these compounds increased after digestion, perhaps due to the acidic conditions of the stomach, which are similar to those found during the oil extraction process. However, after ingestion, plant lignans are partly metabolized by human colonic bacteria before they are absorbed to form the mammalian lignans enterodiol and enterolactone (Heinonen et al., 2001). Conversion of pinoresinol to these enterolignans was approximately 55% in an *in vitro* metabolic model using human faecal microflora (Heinonen et al., 2001), and limited metabolism of phenols was also observed using human colon Caco-2/TC7 cells (Soler et al., 2010). In contrast, Nurmi et al. (2010) noted that pinoresinol should be extensively metabolized considering that only 5% of its intake was detected in the urine of Finnish men. In plasma samples, Suarez et al. (2009) quantified 1-acetoxypinoresinol at 60 and 120 min after the consumption of 30 mL of VOO, but they only found the native form (0.016 μM) in one of five subjects. However, the initial concentrations in the VOO used in the study are unknown, and it is difficult to attribute these results to the metabolism of the compound. On the other hand, the low number of subjects and the high variability observed among them make it impossible to reach conclusions about the concentrations reported.

In summary, metabolism of these plant lignans is not yet well characterized and varies between individuals depending on their diet, the source of the lignan, the expression of the metabolizing enzymes and the intestinal microflora, whose activity can vary from person to person and from day to day (Nurmi et al., 2010). Additionally, many other factors can affect circulating lignan levels in the body such as smoking, antibiotics, obesity (Adlercreutz, 2007) and the techniques used to analyse these levels, including the extraction method or time point of blood collection (Penalvo et al., 2004).

5.3. *Bioavailability*

Very little is known about the availability of (+)-pinoresinol and 1-acetoxypinoresinol in tissues or organs. However, a study carried out using oral administration of the tritium-labelled, dietary lignan secoisolariciresinol diglucoside (3H-SDG) could shed light on the accessibility of lignans to tumour cells, as tumour tissue radioactivity was up 92% in the serum of mice bearing MCF-7 breast cancer xenografts (Saarinen, Power, Chen, & Thompson, 2008). On the other hand, it is not known if these compounds exert their actions as a result of cellular internalization or if the mechanism of action could be reproduced outside of the cell through signalling pathways.

Owen et al. (2000c) proposed that because these lignans are fat soluble, a considerable proportion is likely to be absorbed

Table 3 – Bioactivity of (+)-pinoresinol and 1-acetoxypinoresinol.

Compound	Activity	Form of action	Model	Reference
(+)-pinoresinol	Antitumor	FASN reduction	SKBR3 and MCF7 human breast cancer cells	(Menendez et al., 2008b)
		Decrease on HER2 protein expression and HER2 tyrosine kinase	MCF10A /HER2 human mammary epithelial cells	(Menendez et al., 2009)
		Cytotoxicity	MCF7 human breast cancer, LNCaP human prostate cancer, Lu1 and A549 human lung cancer, KB HeLa derivative, OK-OV-3 human ovarian cancer, MEL-2 human skin melanoma, HCT15 human colon cancer cells.	(Chin et al., 2006; Chiung et al., 1994; Kwak et al., 2009; Sepporta et al., 2013)
		Anti-proliferation, Induction of $p21^{WAF1/Cip1}$ expression	MDA-MB-231 human breast cancer cells, HL60 human leukaemia cells	(Sepporta et al., 2013)
		Anti-invasiveness	HT115 human colon cancer cells	(Hashim et al., 2008)
		Reduction of breast cancer risk	Case-control study	(Torres-Sanchez et al., 2009)
	Antifungal	Inhibition of growth and trichothecene biosynthesis	*Fusarium verticilloides, Fusarium graminearum, Candida albicans, Trichosporon beigelii, Malassezia furfur*	(Hwang et al., 2010; Kulik et al., 2014)
	Antioxidant	Decrease of LDL and liposomes oxidation	*In vitro*	(Kang et al., 2000; Vuorela et al., 2005)
		Translocation of DAF-16	*C. elegans*	(Koch et al., 2015)
		ROS reduction	Male Wistar rats	(Lapi et al., 2015)
	Anti-inflammatory	Inhibition of NO, PGE_2, COX-2, TNFα, IL1β, IL-6 and NF-κβ	J774 murine macrophages, Microglia of rats, Caco-2 human colon cancer cells, RAW264.7 murine macrophages	(During et al., 2012; In et al., 2015; Jung et al., 2010; Lee et al., 2015; Liu et al., 2015; Vuorela et al., 2005; Yang et al., 2013)
		Inhibition of TNFα, iNOS and COX-2. Transactivation of NF-κβ.	Male ICR mice	(Kim et al., 2010)
	Neuroprotective	Cell viability and protective effect against induced cell death	HT22 mouse hippocampal cells	(In et al., 2015)
		Protection on microvascular damage induced, vasodilation.	Male Wistar rats	(Lapi et al., 2015)
	Hypoglycaemic	Inhibition of α-glucosidase	Rat small intestine	(Wikul et al., 2012)
1-acetoxypinoresinol	Antitumor	FASN reduction	SKBR3 and MCF7 breast cancer cells	(Menendez et al., 2008a)
		Decrease on HER2 protein and HER2 tyrosine kinase	MCF10A /HER2 mammary epithelial cells	(Menendez et al., 2009)

and should exert effects on breast cancer and other diseases. Furthermore, the unabsorbed remainder will reach the large intestines where it can exert a chemopreventive effect against colorectal cancer.

Even though it has been shown that 55–73% of ingested olive oil phenols are absorbed (Vissers, Zock, Roodenburg, Leenen, & Katan, 2002), there is limited information about the cellular uptake of the compounds considered in the present review.

According to Garcia-Villalba et al. (2012), approximately 50% of the tested amount of pinoresinol was absorbed by JIMT-1 human breast cancer cells between 2 and 24 h after exposure. The absorption of 1-acetoxypinoresinol (approximately 40%) started within 1 h. In addition, During et al. (2012) showed that pinoresinol can be taken up by intestinal cells. After a 4 h exposure at a final concentration of approximately 40 μM, human colon adenocarcinoma Caco-2 cells took up 2% of the pinoresinol, from which 75% was in a conjugated form. The cellular uptake of free pinoresinol (6 to 259 μM) increased linearly with the initial concentration, indicating that it entered cells by simple diffusion or with the help of a low affinity transporter. In contrast, they showed that lignan conjugation is a saturable process, because conjugated forms first increased proportionally to the initial concentration and then plateaued at approximately 30–40 μM (During et al., 2012). The fact that a

specific transporter is not required by cells for pinoresinol uptake indicates that this compound may be available for uptake by other cell types in which it could also exert its biological effects.

As reported by Sepporta et al. (2013), although plasma concentrations of lignans exist in the nanomolar range, tissue concentrations remain unknown and bioaccumulation phenomena cannot be excluded.

It is worth noting that the interaction between polyphenols and some food components such as proteins, carbohydrates, fibre, fat and alcohol, could affect their absorption, elimination and half-life in the blood (reviewed by D'Archivio, Filesi, Vari, Scazzocchio, & Masella, 2010). However, no references have discussed how these interactions or conjugation affect pinoresinol and 1-acetoxypinoresinol, and further investigation is needed.

6. Prospects

It has been reported that pinoresinol and 1-acetoxypinoresinol have several health benefit, from free radical scavenging activity to biological effects *in vitro* and *in vivo*. They have been associated with antitumor activity, especially in breast tumours, and with a decrease in the expression of proteins and molecules, such FASN and HER2 tyrosine kinase, that are directly implicated in cancer processes. Both compounds also possess chemical antioxidant activity. However, little data have been published about the effects of 1-acetoxypinoresinol and, taking into account the preliminary information known about this lignan, it would be worth further study.

References also suggest that (+)-pinoresinol exerts anti-inflammatory activity, specifically through the inhibition of NO but also through the inhibition of inflammatory mediators such as PGE2, COX-2, TNFα, IL1β, IL-6 and NF-κβ. Therefore, consumption of this compound could be clinically applied to target inflammatory processes, which are the basis for the initiation of several diseases such as cancer.

Furthermore, this compound has shown antifungal effects against several human pathogenic fungi, as well as neuroprotective and hypoglycaemic actions. Finally, pinoresinol's antioxidant activity, reported by a few authors who used DPPH and ABTS assays for analysis, seems to be effective both *in vitro* and *in vivo*.

Due to the potential pharmacological properties inherent within these lignans, further investigation is needed in order to determine their effects and mechanisms of action. Moreover, it would be crucial to elucidate currently unknown aspects such as their concentrations in foods, their ability to be metabolized and whether cells or tissues can efficiently absorb them in their native form, including in what concentrations. However, it appears that these compounds remain stable during digestion, and there is a high probably that they enter cells by simple diffusion, indicating their potential availability to nearly all tissues.

Thus, more studies are necessary in order to better understand these compounds, especially given that both (+)-pinoresinol and 1-acetoxypinoresinol have broad pharmacological potential as anti-tumour, anti-inflammatory, antioxidant and antifungal agents.

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Conflict of interest

The authors declare no competing financial or personal interest.

REFERENCES

Adlercreutz, H. (2007). Lignans and human health. *Critical Reviews in Clinical Laboratory Sciences*, *44*(5–6), 483–525.

Antonini, E., Farina, A., Leone, A., Mazzara, E., Urbani, S., Selvaggini, R., Servili, M., & Ninfali, P. (2015). Phenolic compounds and quality parameters of family farming versus protected designation of origin (PDO) extra-virgin olive oils. *Journal of Food Composition and Analysis*, *43*, 75–81. doi:10.1016/j.jfca.2015.04.015.

Arslan, D., Karabekir, Y., & Schreiner, M. (2013). Variations of phenolic compounds, fatty acids and some qualitative characteristics of Sarıulak olive oil as induced by growing area. *Food Research International*, *54*(2), 1897–1906. doi:10.1016/j.foodres.2013.06.016.

Bonoli, M., Bendini, A., Cerretani, L., Lercker, G., & Toschi, T. G. (2004). Qualitative and semiquantitative analysis of phenolic compounds in extra virgin olive oils as a function of the ripening degree of olive fruits by different analytical techniques. *Journal of Agricultural and Food Chemistry*, *52*(23), 7026–7032. doi:10.1021/jf048868m.

Brenes, M., García, A., Rios, J. J., García, P., & Garrido, A. (2002b). Use of 1-acetoxypinoresinol to authenticate Picual olive oils. *International Journal of Food Science & Technology*, *37*(6), 615–625. doi:10.1046/j.1365-2621.2002.00591.x.

Brenes, M., Garcia, A., Dobarganes, M. C., Velasco, J., & Romero, C. (2002a). Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. *Journal of Agricultural and Food Chemistry*, *50*(21), 5962–5967.

Brenes, M., Garcia, A., Garcia, P., & Garrido, A. (2001). Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. *Journal of Agricultural and Food Chemistry*, *49*(11), 5609–5614.

Brenes, M., Hidalgo, F., Garcia, A., Rios, J., Garcia, P., Zamora, R., & Garrido, A. (2000). Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *Journal of the American Oil Chemists' Society*, *77*(7), 715–720.

Carpinella, M. C., Giorda, L. M., Ferrayoli, C. G., & Palacios, S. M. (2003). Antifungal effects of different organic extracts from Melia azedarach L. on phytopathogenic fungi and their isolated active components. *Journal of Agricultural and Food Chemistry*, *51*(9), 2506–2511. doi:10.1021/jf026083f.

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Del Carlo, M., Gallina-Toschi, T., Lercker, G., Compagnone, D., & Fernandez-Gutierrez, A. (2005). Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *Journal of Agricultural and Food Chemistry*, *53*(23), 8918–8925. doi:10.1021/jf0515680.

Chin, Y. W., Chai, H. B., Keller, W. J., & Kinghorn, A. D. (2008). Lignans and other constituents of the fruits of Euterpe oleracea (acai) with antioxidant and cytoprotective activities.

Journal of Agricultural and Food Chemistry, 56(17), 7759–7764. doi:10.1021/jf801792n.

Chin, Y. W., Jones, W. P., Rachman, I., Riswan, S., Kardono, L. B., Chai, H. B., Farnsworth, N. R., Cordell, G. A., Swanson, S. M., Cassady, J. M., & Kinghorn, A. D. (2006). Cytotoxic lignans from the stems of Helicteres hirsuta collected in Indonesia. *Phytotherapy Research : PTR*, *20*(1), 62–65. doi:10.1002/ptr.1806.

Chiung, Y. M., Hayashi, H., Matsumoto, H., Otani, T., Yoshida, K., Huang, M. Y., Chen, R. X., Liu, J. R., & Nakayama, M. (1994). New metabolites, tetrahydrofuran lignans, produced by streptomyces sp. IT-44. *Journal of Antibiotics*, *47*(4), 487–491.

Chtourou, M., Gargouri, B., Jaber, H., Abdelhedi, R., & Bouaziz, M. (2013). Comparative study of olive oil quality from Chemlali Afax versus Arbequina cultivated in Tunisia. *European Journal of Lipid Science and Technology : EJLST*, *115*(6), 631–640. doi:10.1002/ejlt.201200234.

Cotterchio, M., Boucher, B. A., Kreiger, N., Mills, C. A., & Thompson, L. U. (2008). Dietary phytoestrogen intake – lignans and isoflavones – and breast cancer risk (Canada). *Cancer Causes & Control : CCC*, *19*(3), 259–272. doi:10.1007/s10552-007-9089-2.

Dar, A. A., & Arumugam, N. (2013). Lignans of sesame: Purification methods, biological activities and biosynthesis – A review. *Bioorganic Chemistry*, *50*, 1–10. doi:10.1016/j.bioorg.2013.06.009.

D'Archivio, M., Filesi, C., Vari, R., Scazzocchio, B., & Masella, R. (2010). Bioavailability of the polyphenols: Status and controversies. *International Journal of Molecular Sciences*, *11*(4), 1321–1342. doi:10.3390/ijms11041321.

During, A., Debouche, C., Raas, T., & Larondelle, Y. (2012). Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal caco-2 cells. *The Journal of Nutrition*, *142*(10), 1798–1805.

Fini, L., Hotchkiss, E., Fogliano, V., Graziani, G., Romano, M., De Vol, E. B., Qin H., Selgrad M., Boland C. R., & Ricciardiello, L. (2008). Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. *Carcinogenesis*, *29*(1), 139–146.

Gambacorta, G., Faccia, M., Previtali, M. A., Pati, S., La Notte, E., & Baiano, A. (2010). Effects of olive maturation and stoning on quality indices and antioxidant content of extra virgin oils (cv. Coratina) during storage. *Journal of Food Science*, *75*(3), C229–C235. doi:10.1111/j.1750-3841.2010.01516.x.

García, A., Brenes, M., Martínez, F., Alba, J., García, P., & Garrido, A. (2001). High-performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale. *Journal of the American Oil Chemists' Society*, *78*(6), 625–629.

Garcia-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Menendez, J. A., Segura-Carretero, A., & Fernandez-Gutierrez, A. (2012). Uptake and metabolism of olive oil polyphenols in human breast cancer cells using nano-liquid chromatography coupled to electrospray ionization-time of flight-mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *898*, 69–77. doi:10.1016/j.jchromb.2012.04.021.

Gómez Caravaca, A. M., Carrasco Pancorbo, A., Canabate Diaz, B., Segura Carretero, A., & Fernandez Gutierrez, A. (2005). Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil. *Electrophoresis*, *26*(18), 3538–3551. doi:10.1002/elps.200500202.

Harborne, J. B., Baxter, H., & Moss, G. P. (1999). *Phytochemical dictionary: A handbook of bioactive compounds from plants*. Wallington: Taylor & Francis.

Hashim, Y. Z., Rowland, I. R., McGlynn, H., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., Montedoro, G., Kaisalo, L., Wähälä, K., & Gill, C. I. (2008). Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. *International Journal of Cancer. Journal International du Cancer*, *122*(3), 495–500. doi:10.1002/ijc.23148.

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wahala, K., Deyama, T., Nishibe, S., & Adlercreutz, H. (2001). In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol. *Journal of Agricultural and Food Chemistry*, *49*(7), 3178–3186.

Hirano, T., Fukuoka, K., Oka, K., Naito, T., Hosaka, K., Mitsuhashi, H., & Matsumoto, Y. (1990). Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. *Cancer Investigation*, *8*(6), 595–602.

Hwang, B., Lee, J., Liu, Q. H., Woo, E. R., & Lee, D. G. (2010). Antifungal effect of (+)-pinoresinol isolated from Sambucus williamsii. *Molecules (Basel, Switzerland)*, *15*(5), 3507–3516. doi:10.3390/molecules15053507.

In, S. J., Seo, K. H., Song, N. Y., Lee, D. S., Kim, Y. C., & Baek, N. I. (2015). Lignans and neolignans from the stems of Viburnum erosum and their neuroprotective and anti-inflammatory activity. *Archives of Pharmacal Research*, *38*(1), 26–34. doi:10.1007/s12272-014-0358-9.

Jung, H. W., Mahesh, R., Lee, J. G., Lee, S. H., Kim, Y. S., & Park, Y. K. (2010). Pinoresinol from the fruits of forsythia koreana inhibits inflammatory responses in LPS-activated microglia. *Neuroscience Letters*, *480*(3), 215–220. doi:10.1016/j.neulet.2010.06.043.

Kajla, P., Sharma, A., & Sood, D. R. (2015). Flaxseed-a potential functional food source. *Journal of Food Science and Technology*, *52*(4), 1857–1871. doi:10.1007/s13197-014-1293-y.

Kalua, C. M., Bedgood, D. R., Jr., Bishop, A. G., & Prenzler, P. D. (2008). Changes in virgin olive oil quality during low-temperature fruit storage. *Journal of Agricultural and Food Chemistry*, *56*(7), 2415–2422. doi:10.1021/jf073027b.

Kang, M. H., Naito, M., Sakai, K., Uchida, K., & Osawa, T. (2000). Mode of action of sesame lignans in protecting low-density lipoprotein against oxidative damage in vitro. *Life Sciences*, *66*(2), 161–171.

Kang, W., & Wang, J. (2010). In vitro antioxidant properties and in vivo lowering blood lipid of forsythia suspense leaves. *Medicinal Chemistry Research*, *19*, 617–628.

Kardono, L. B., Tsauri, S., Padmawinata, K., Pezzuto, J. M., & Kinghorn, A. D. (1990). Cytotoxic constituents of the bark of Plumeria rubra collected in Indonesia. *Journal of Natural Products*, *53*(6), 1447–1455.

Kim, H. J., Ono, E., Morimoto, K., Yamagaki, T., Okazawa, A., Kobayashi, A., & Satake, H. (2009). Metabolic engineering of lignan biosynthesis in forsythia cell culture. *Plant & Cell Physiology*, *50*(12), 2200–2209. doi:10.1093/pcp/pcp156.

Kim, H. Y., Kim, J. K., Choi, J. H., Jung, J. Y., Oh, W. Y., Kim, D. C., Lee, H. S., Kim, Y. S., Kang, S. S., Lee, S. H., & Lee, S. M. (2010). Hepatoprotective effect of pinoresinol on carbon tetrachloride-induced hepatic damage in mice. *Journal of Pharmacological Sciences*, *112*(1), 105–112.

Koch, K., Buchter, C., Havermann, S., & Watjen, W. (2015). The lignan pinoresinol induces nuclear translocation of DAF-16 in Caenorhabditis elegans but has no effect on life span. *Phytotherapy Research : PTR*, *29*(6), 894–901. doi:10.1002/ptr.5330.

Kulik, T., Busko, M., Pszczolkowska, A., Perkowski, J., & Okorski, A. (2014). Plant lignans inhibit growth and trichothecene biosynthesis in Fusarium graminearum. *Letters in Applied Microbiology*, *59*(1), 99–107. doi:10.1111/lam.12250.

Kuo, P. C., Lin, M. C., Chen, G. F., Yiu, T. J., & Tzen, J. T. (2011). Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. *Journal of Agricultural and Food Chemistry*, *59*(7), 3214–3219. doi:10.1021/jf104311g.

Kwak, J. H., Kang, M. W., Roh, J. H., Choi, S. U., & Zee, O. P. (2009). Cytotoxic phenolic compounds from Chionanthus retusus.

Archives of Pharmacal Research, *32*(12), 1681–1687. doi:10.1007/s12272-009-2203-0.

Lapi, D., Di Maro, M., Mastantuono, T., Battiloro, L., Sabatino, L., Muscariello, E., & Colantuoni, A. (2015). Effects of oleuropein and pinoresinol on microvascular damage induced by hypoperfusion and reperfusion in rat pial circulation. *Microcirculation (New York, N.Y.: 1994)*, *22*(1), 79–90. doi:10.1111/micc.12175.

Lee, J. W., Lee, J. H., Lee, C., Jin, Q., Lee, D., Kim, Y., Hong, J. T., Lee, M. K., & Hwang, B. Y. (2015). Inhibitory constituents of Sophora tonkinensis on nitric oxide production in RAW 264.7 macrophages. *Bioorganic & Medicinal Chemistry Letters*, *25*(4), 960–962. doi:10.1016/j.bmcl.2014.12.012.

Li, C. Y., Zhai, H. Y., Tang, S. A., & Duan, H. Q. (2010). Studies on the chemical constituents of Pachysandra terminalis and their antioxidant activity. *Journal of Chinese Medicinal Materials*, *33*(5), 729–732.

Lin, Y., Yngve, A., Lagergren, J., & Lu, Y. (2012). Dietary intake of lignans and risk of adenocarcinoma of the esophagus and gastroesophageal junction. *Cancer Causes & Control : CCC*, *23*(6), 837–844. doi:10.1007/s10552-012-9952-7.

Liu, J., Nakamura, S., Xu, B., Matsumoto, T., Ohta, T., Fujimoto, K., Ogawa, K., Fukaya, M., Miyake, S., & Matsuda, H. (2015). Chemical structures of constituents from the flowers of Osmanthus fragrans var. aurantiacus. *Journal of Natural Medicines*, *69*(1), 135–141. doi:10.1007/s11418-014-0869-1.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727–747.

Menendez, J. A., Vazquez-Martin, A., Garcia-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Fernandez-Gutierrez, A., & Segura-Carretero, A. (2008b). tabAnti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial extra-virgin olive oil (EVOO). *BMC Cancer*, *8*, 377. doi:10.1186/1471-2407-8-377.

Menendez, J. A., Vazquez-Martin, A., Oliveras-Ferraros, C., Garcia-Villalba, R., Carrasco-Pancorbo, A., Fernandez-Gutierrez, A., & Segura-Carretero, A. (2008a). Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. *International Journal of Molecular Medicine*, *22*(4), 433–439.

Menendez, J. A., Vazquez-Martin, A., Oliveras-Ferraros, C., Garcia-Villalba, R., Carrasco-Pancorbo, A., Fernandez-Gutierrez, A., & Segura-Carretero, A. (2009). Extra-virgin olive oil polyphenols inhibit HER2 (erbB-2)-induced malignant transformation in human breast epithelial cells: Relationship between the chemical structures of extra-virgin olive oil secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2. *International Journal of Oncology*, *34*(1), 43–51.

Milder, I. E., Feskens, E. J., Arts, I. C., Bueno de Mesquita, H. B., Hollman, P. C., & Kromhout, D. (2005). Intake of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in Dutch men and women. *The Journal of Nutrition*, *135*(5), 1202–1207.

Mimaki, Y., Kuroda, M., Asano, T., & Sashida, Y. (1999). Triterpene saponins and lignans from the roots of Pulsatilla chinensis and their cytotoxic activity against HL-60 cells. *Journal of Natural Products*, *62*(9), 1279–1283.

Minh, C. V., Nhiem, N. X., Yen, H. T., Kiem, P. V., Tai, B. H., Le Tuan Anh, H., Hien, T. T., Park, S., Kim, N., & Kim, S. H. (2015). Chemical constituents of Trichosanthes kirilowii and their cytotoxic activities. *Archives of Pharmacal Research*, *38*(8), 1443–1448. doi:10.1007/s12272-014-0490-6.

Nurmi, T., Mursu, J., Peñalvo, J. L., Poulsen, H. E., & Voutilainen, S. (2010). Dietary intake and urinary excretion of lignans in Finnish men. *The British Journal of Nutrition*, *103*(05), 677–685. doi:10.1017/S0007114509992261.

Oliveras López, M. J., Innocenti, M., Ieri, F., Giaccherini, C., Romani, A., & Mulinacci, N. (2008). HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian Olea europaea L. fruits. *Journal of Food Composition and Analysis*, *21*(1), 62–70. doi:10.1016/j.jfca.2007.04.012.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B., & Bartsch, H. (2000a). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer (Oxford, England: 1990)*, *36*(10), 1235–1247.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Wurtele, G., Spiegelhalder, B., & Bartsch, H. (2000c). Olive-oil consumption and health: The possible role of antioxidants. *The Lancet Oncology*, *1*, 107–112.

Owen, R. W., Mier, W., Giacosa, A., Hull, W. E., Spiegelhalder, B., & Bartsch, H. (2000b). Identification of lignans as major components in the phenolic fraction of olive oil. *Clinical Chemistry*, *46*(7), 976–988.

Pan, J. Y., Chen, S. L., Yang, M. H., Wu, J., Sinkkonen, J., & Zou, K. (2009). An update on lignans: Natural products and synthesis. *Natural Product Reports*, *26*(10), 1251–1292. doi:10.1039/b910940d.

Penalvo, J. L., Nurmi, T., Haajanen, K., Al-Maharik, N., Botting, N., & Adlercreutz, H. (2004). Determination of lignans in human plasma by liquid chromatography with coulometric electrode array detection. *Analytical Biochemistry*, *332*(2), 384–393. doi:10.1016/j.ab.2004.05.046.

Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., & McCullough, M. L. (2010). Dietary lignans: Physiology and potential for cardiovascular disease risk reduction. *Nutrition Reviews*, *68*(10), 571–603. doi:10.1111/j.1753-4887.2010.00319.x.

Rafieian-Kopaei, M., Setorki, M., Doudi, M., Baradaran, A., & Nasri, H. (2014). Atherosclerosis: Process, indicators, risk factors and new hopes. *International Journal of Preventive Medicine*, *5*(8), 927–946.

Saarinen, N. M., Power, K. A., Chen, J., & Thompson, L. U. (2008). Lignans are accessible to human breast cancer xenografts in athymic mice. *Nutrition and Cancer*, *60*(2), 245–250. doi:10.1080/01635580701649669.

Saleem, M., Kim, H. J., Ali, M. S., & Lee, Y. S. (2005). An update on bioactive plant lignans. *Natural Product Reports*, *22*(6), 696–716. doi:10.1039/b514045p.

Selvaggini, R., Servili, M., Urbani, S., Esposto, S., Taticchi, A., & Montedoro, G. (2006). Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection. *Journal of Agricultural and Food Chemistry*, *54*(8), 2832–2838. doi:10.1021/jf0527596.

Sepporta, M. V., Mazza, T., Morozzi, G., & Fabiani, R. (2013). Pinoresinol inhibits proliferation and induces differentiation on human HL60 leukemia cells. *Nutrition and Cancer*, *65*(8), 1208–1218. doi:10.1080/01635581.2013.828089.

Servili, M., Taticchi, A., Esposito, S., Urbani, S., Selvaggini, R., & Montedoro, G. (2007). Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. *Journal of Agricultural and Food Chemistry*, *55*(17), 7028–7035. doi:10.1021/jf070600i.

Shi, Y. N., Shi, Y. M., Yang, L., Li, X. C., Zhao, J. H., Qu, Y., Zhu, H. T., Wang, D., Cheng, R. R., Yang, C. R., Xu, M., & Zhang, Y. J. (2015). Lignans and aromatic glycosides from Piper wallichii and their antithrombotic activities. *Journal of Ethnopharmacology*, *162*, 87–96. doi:10.1016/j.jep.2014.12.038.

Sok, D. E., Cui, H. S., & Kim, M. R. (2009). Isolation and bioactivities of furfuran type lignan compounds from edible plants. *Recent Patents on Food, Nutrition & Agriculture*, *1*(1), 87–95.

Soler, A., Romero, M. P., Macià, A., Saha, S., Furniss, C. S. M., Kroon, P. A., & Motilva, M. J. (2010). Digestion stability and evaluation of the metabolism and transport of olive oil

phenols in the human small-intestinal epithelial caco-2/TC7 cell line. *Food Chemistry*, 119, 703–714.
Suarez, M., Romero, M. P., Macia, A., Valls, R. M., Fernandez, S., Sola, R., & Motilva, M. J. (2009). Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *877*(32), 4097–4106. doi:10.1016/j.jchromb.2009.10.025.
Thompson, L. U., Chen, J. M., Li, T., Strasser-Weippl, K., & Goss, P. E. (2005). Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *11*(10), 3828–3835.
Toledo, E., Salas-Salvado, J., Donat-Vargas, C., Buil-Cosiales, P., Estruch, R., Ros, E., Corella, D., Fitó, M., Hu, F. B., Arós, F., Gómez-Gracia, E., Romaguera, D., Ortega-Calvo, M., Serra-Majem, L., Pintó, X., Schröder, H., Basora, J., Sorlí, J. V., Bulló, M., Serra-Mir, M., & Martinez-Gonzalez, M. A. (2015). Mediterranean diet and invasive breast cancer risk among women at high cardiovascular risk in the PREDIMED trial: A randomized clinical trial. *JAMA Internal Medicine*, 175(11), 1752–1760. doi:10.1001/jamainternmed.2015.4838.
Torres-Sanchez, L., Galvan-Portillo, M., Wolff, M. S., & Lopez-Carrillo, L. (2009). Dietary consumption of phytochemicals and breast cancer risk in Mexican women. *Public Health Nutrition*, *12*(6), 825–831. doi:10.1017/S136898000800325X.
Touillaud, M. S., Thiebaut, A. C., Fournier, A., Niravong, M., Boutron-Ruault, M. C., & Clavel-Chapelon, F. (2007). Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. *Journal of the National Cancer Institute*, *99*(6), 475–486.
Tsukamoto, H., Hisada, S., & Nishibe, S. (1984). Lignans from bark of the Olea plants. I. *Chemical & Pharmaceutical Bulletin*, *32*(7), 2730–2735.
Umezawa, T. (2003). Diversity in lignan biosynthesis. *Phytochemistry Reviews*, *2*(3), 371–390.
Vissers, M. N., Zock, P. L., Roodenburg, A. J., Leenen, R., & Katan, M. B. (2002). Olive oil phenols are absorbed in humans. *The Journal of Nutrition*, *132*(3), 409–417.
Vuorela, S., Kreander, K., Karonen, M., Nieminen, R., Hamalainen, M., Galkin, A., Laitinen, L., Salminen, J. P., Moilanen, E., Pihlaja, K., Vuorela, H., Vuorela, P., & Heinonen, M. (2005). Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *Journal of Agricultural and Food Chemistry*, *53*(15), 5922–5931. doi:10.1021/jf050554r.
Wikul, A., Damsud, T., Kataoka, K., & Phuwapraisirisan, P. (2012). (+)-Pinoresinol is a putative hypoglycemic agent in defatted sesame (Sesamum indicum) seeds though inhibiting alpha-glucosidase. *Bioorganic & Medicinal Chemistry Letters*, *22*(16), 5215–5217. doi:10.1016/j.bmcl.2012.06.068.
Yang, C. P., Huang, G. J., Huang, H. C., Chen, Y. C., Chang, C. I., Wang, S. Y., Chang, H. S., Tseng, Y. H., Chien, S. C., & Kuo, Y. H. (2013). The effect of the aerial part of Lindera akoensis on lipopolysaccharides (LPS)-induced nitric oxide production in RAW264.7 cells. *International Journal of Molecular Sciences*, *14*(5), 9168–9181. doi:10.3390/ijms14059168.
Yeo, H., Chin, Y. W., Park, S. Y., & Kim, J. (2004). Lignans of Rosa multiflora roots. *Archives of Pharmacal Research*, *27*(3), 287–290.
Yi, B., Hu, L., Mei, W., Zhou, K., Wang, H., Luo, Y., Wei X., & Dai, H. (2011). Antioxidant phenolic compounds of cassava (Manihot esculenta) from Hainan. *Molecules (Basel, Switzerland)*, *16*(12), 10157–10167. doi:10.3390/molecules161210157.

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Review

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

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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

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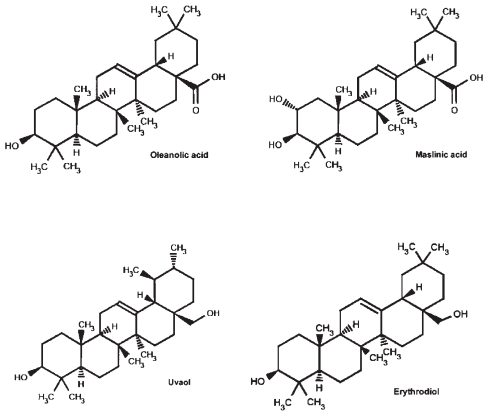

Figure 1. Chemical structures of oleanolic and maslinic acids and uvaol and erythrodiol dialcohols of olive oil.

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

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

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

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

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

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

BIOACTIVE PROPERTIES OF OLIVE OIL TRITERPENES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

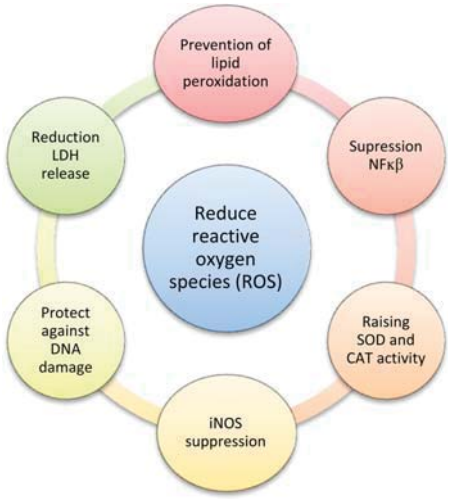

Figure 2. Oxidative mechanisms affected by triterpenes.

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

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

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

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

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

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

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

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

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

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

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

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

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REFERENCES

(1) Klonizakis, M.; Alkhatib, A.; Middleton, G.; Smith, M. F. Mediterranean diet- and exercise-induced improvement in age-dependent vascular activity. *Clin. Sci.* **2013**, *124*, 579−587.

(2) Mitjavila, M. T.; Fandos, M.; Salas-Salvadó, J.; Covas, M.; Borrego, S.; Estruch, R.; Lamuela-Raventós, R.; Corella, D.; Martínez-Gonzalez, M. T.; Sánchez, J. M.; Bulló, M.; Fitó, M.; Tormos, C.; Cerdá, C.; Casillas, R.; Moreno, J. J.; Iradi, A.; Zaragoza, C.; Chaves, J.; Sáez, G. T. The Mediterranean diet improves the systemic lipid and DNA oxidative damage in metabolic syndrome individuals. A randomized, controlled, trial. *Clin. Nutr.* **2013**, *32*, 172−178.

(3) De Lorgeril, M.; Renaud, S.; Mamelle, N.; Salen, P.; Martin, J.-.; Monjaud, I.; Guidollet, J.; Touboul, P.; Delaye, J. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet* **1994**, *343*, 1454−1459.

(4) Willett, W. C.; Sacks, F.; Trichopoulou, A.; Drescher, G.; Ferro-Luzzi, A.; Helsing, E.; Trichopoulos, D. Mediterranean diet pyramid: a cultural model for healthy eating. *Am. J. Clin. Nutr.* **1995**, *61*, 1402S−1406S.

(5) Covas, M. I. Olive oil and the cardiovascular system. *Pharmacol. Res.* **2007**, *55*, 175−186.

(6) Allouche, Y.; Beltran, G.; Gaforio, J. J.; Uceda, M.; Mesa, M. D. Antioxidant and antiatherogenic activities of pentacyclic triterpenic diols and acids. *Food Chem. Toxicol.* **2010**, *48*, 2885−2890.

(7) Allouche, Y.; Warleta, F.; Campos, M.; Sanchez-Quesada, C.; Uceda, M.; Beltran, G.; Gaforio, J. J. 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.* **2011**, *59*, 121−130.

(8) Warleta, F.; Quesada, C. S.; Campos, M.; Allouche, Y.; Beltran, G.; Gaforio, J. J. Hydroxytyrosol protects against oxidative DNA damage in human breast cells. *Nutrients* **2011**, *3*, 839−857.

(9) Warleta, F.; Campos, M.; Allouche, Y.; Sánchez-Quesada, C.; Ruiz-Mora, J.; Beltrán, G.; Gaforio, J. J. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. *Food Chem. Toxicol.* **2010**, *48*, 1092−1100.

(10) Huang, L.; Guan, T.; Qian, Y.; Huang, M.; Tang, X.; Li, Y.; Sun, H. Anti-inflammatory effects of maslinic acid, a natural triterpene, in cultured cortical astrocytes via suppression of nuclear factor-kappa B. *Eur. J. Pharmacol.* **2011**, *672*, 169−174.

(11) Allouche, Y.; Jimenez, A.; Uceda, M.; Aguilera, M. P.; Gaforio, J. J.; Beltran, G. Triterpenic content and chemometric analysis of virgin olive oils from forty olive cultivars. *J. Agric. Food Chem.* **2009**, *57*, 3604−3610.

(12) Perez-Camino, M. C.; Cert, A. Quantitative determination of hydroxy pentacyclic triterpene acids in vegetable oils. *J. Agric. Food Chem.* **1999**, *47*, 1558−1562.

(13) Juan, M. E.; Wenzel, U.; Ruiz-Gutierrez, V.; Daniel, H.; Planas, J. M. Olive fruit extracts inhibit proliferation and induce apoptosis in HT-29 human colon cancer cells. *J. Nutr.* **2006**, *136*, 2553−2557.

(14) Lu, Y. F.; Wan, X. L.; Xu, Y.; Liu, J. Repeated oral administration of oleanolic acid produces cholestatic liver injury in mice. *Molecules* **2013**, *18*, 3060−3071.

(15) Marquez-Martin, A.; De La Puerta, R.; Fernandez-Arche, A.; Ruiz-Gutierrez, V.; Yaqoob, P. Modulation of cytokine secretion by pentacyclic triterpenes from olive pomace oil in human mononuclear cells. *Cytokine* **2007**, *36*, 211−217.

(16) Moreno, J. J. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radical Biol. Med.* **2003**, *35*, 1073−1081.

(17) Brunelleschi, S.; Bardelli, C.; Amoruso, A.; Gunella, G.; Ieri, F.; Romani, A.; Malorni, W.; Franconi, F. Minor polar compounds extra-virgin olive oil extract (MPC-OOE) inhibits NF-kappa B translocation in human monocyte/macrophages. *Pharmacol. Res.* **2007**, *56*, 542−549.

(18) Giner-Larza, E. M.; Manez, S.; Recio, M. C.; Giner, R. M.; Prieto, J. M.; Cerda-Nicolas, M.; Rios, J. L. Oleanonic acid, a 3-oxotriterpene from *Pistacia*, inhibits leukotriene synthesis and has anti-inflammatory activity. *Eur. J. Pharmacol.* **2001**, *428*, 137−143.

(19) Martin, R.; Ibeas, E.; Carvalho-Tavares, J.; Hernandez, M.; Ruiz-Gutierrez, V.; Nieto, M. L. Natural triterpenic diols promote apoptosis in astrocytoma cells through ROS-mediated mitochondrial depolarization and JNK activation. *PLoS One* **2009**, *4*, e5975.

(20) De Caterina, R.; Zampolli, A.; Del Turco, S.; Madonna, R.; Massaro, M. Nutritional mechanisms that influence cardiovascular disease. *Am. J. Clin. Nutr.* **2006**, *83*, 421S−426S.

(21) Holvoet, P.; Harris, T. B.; Tracy, R. P.; Verhamme, P.; Newman, A. B.; Rubin, S. M.; Simonsick, E. M.; Colbert, L. H.; Kritchevsky, S. B. Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 1444−1448.

(22) Meisinger, C.; Baumert, J.; Khuseyinova, N.; Loewel, H.; Koenig, W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation* **2005**, *112*, 651−657.

(23) Mertens, A.; Verhamme, P.; Bielicki, J. K.; Phillips, M. C.; Quarck, R.; Verreth, W.; Stengel, D.; Ninio, E.; Navab, M.; Mackness, B.; Mackness, M.; Holvoet, P. Increased low-density lipoprotein oxidation and impaired high-density lipoprotein antioxidant defense are associated with increased macrophage homing and atherosclerosis in dyslipidemic obese mice: LCAT gene transfer decreases atherosclerosis. *Circulation* **2003**, *107*, 1640−1646.

(24) Matsuura, E.; Hughes, G. R.; Khamashta, M. A. Oxidation of LDL and its clinical implication. *Autoimmun. Rev.* **2008**, *7*, 558−566.

(25) Lapointe, A.; Couillard, C.; Lemieux, S. Effects of dietary factors on oxidation of low-density lipoprotein particles. *J. Nutr. Biochem.* **2006**, *17*, 645−658.

(26) Ferdowsian, H. R.; Barnard, N. D. Effects of plant-based diets on plasma lipids. *Am. J. Cardiol.* **2009**, *104*, 947−956.

(27) Andrikopoulos, N. K.; Kaliora, A. C.; Assimopoulou, A. N.; Papapeorgiou, V. P. Biological activity of some naturally occurring

resins, gums and pigments against in vitro LDL oxidation. *Phytother. Res.* **2003**, *17*, 501−507.
(28) Andrikopoulos, N. K.; Kaliora, A. C.; Assimopoulou, A. N.; Papageorgiou, V. P. Inhibitory activity of minor polyphenolic and nonpolyphenolic constituents of olive oil against in vitro low-density lipoprotein oxidation. *J. Med. Food* **2002**, *5*, 1−7.
(29) Badimon, J. J.; Ibanez, B.; Cimmino, G. Genesis and dynamics of atherosclerotic lesions: implications for early detection. *Cerebrovasc. Dis.* **2009**, *27* (Suppl. 1), 38−47.
(30) Lusis, A. J. Atherosclerosis. *Nature* **2000**, *407*, 233−241.
(31) Buus, N. H.; Hansson, N. C.; Rodriguez-Rodriguez, R.; Stankevicius, E.; Andersen, M. R.; Simonsen, U. Antiatherogenic effects of oleanolic acid in apolipoprotein E knockout mice. *Eur. J. Pharmacol.* **2011**, *670*, 519−526.
(32) Lou-Bonafonte, J. M.; Arnal, C.; Navarro, M. A.; Osada, J. Efficacy of bioactive compounds from extra virgin olive oil to modulate atherosclerosis development. *Mol. Nutr. Food Res.* **2012**, *56*, 1043−1057.
(33) Hussain Shaik, A.; Rasool, S. N.; Abdul Kareem, M.; Krushna, G. S.; Akhtar, P. M.; Devi, K. L. Maslinic acid protects against isoproterenol-induced cardiotoxicity in albino Wistar rats. *J. Med. Food* **2012**, *15*, 741−746.
(34) Rodriguez-Rodriguez, R.; Herrera, M. D.; de Sotomayor, M. A.; Ruiz-Gutierrez, V. Effects of pomace olive oil-enriched diets on endothelial function of small mesenteric arteries from spontaneously hypertensive rats. *Br. J. Nutr.* **2009**, *102*, 1435−1444.
(35) Rodriguez-Rodriguez, R.; Herrera, M. D.; de Sotomayor, M. A.; Ruiz-Gutierrez, V. Pomace olive oil improves endothelial function in spontaneously hypertensive rats by increasing endothelial nitric oxide synthase expression. *Am. J. Hypertens.* **2007**, *20*, 728−734.
(36) Rodriguez-Rodriguez, R.; Perona, J. S.; Herrera, M. D.; Ruiz-Gutierrez, V. Triterpenic compounds from "orujo" olive oil elicit vasorelaxation in aorta from spontaneously hypertensive rats. *J. Agric. Food Chem.* **2006**, *54*, 2096−2102.
(37) Somova, L. O.; Nadar, A.; Rammanan, P.; Shode, F. O. Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine* **2003**, *10*, 115−121.
(38) Liu, C. H.; Huang, X. T.; Li, Y. Y.; Zheng, X.; Li, N.; Mi, S. Q.; Wang, N. S. The anti-portal hypertension effect of oleanolic acid in CCl_4-induced cirrhosis rats. *Zhong Yao Cai* **2012**, *35*, 930−935.
(39) Bachhav, S. S.; Patil, S. D.; Bhutada, M. S.; Surana, S. J. Oleanolic acid prevents glucocorticoid-induced hypertension in rats. *Phytother. Res.* **2011**, *25*, 1435−1439.
(40) Rodriguez-Rodriguez, R.; Herrera, M. D.; Perona, J. S.; Ruiz-Gutierrez, V. Potential vasorelaxant effects of oleanolic acid and erythrodiol, two triterpenoids contained in 'orujo' olive oil, on rat aorta. *Br. J. Nutr.* **2004**, *92*, 635−642.
(41) Rodriguez-Rodriguez, R.; Stankevicius, E.; Herrera, M. D.; Ostergaard, L.; Andersen, M. R.; Ruiz-Gutierrez, V.; Simonsen, U. Oleanolic acid induces relaxation and calcium-independent release of endothelium-derived nitric oxide. *Br. J. Pharmacol.* **2008**, *155*, 535−546.
(42) Martin, R.; Miana, M.; Jurado-Lopez, R.; Martinez-Martinez, E.; Gomez-Hurtado, N.; Delgado, C.; Bartolome, M. V.; San Roman, J. A.; Cordova, C.; Lahera, V.; Nieto, M. L.; Cachofeiro, V. DIOL triterpenes block profibrotic effects of angiotensin II and protect from cardiac hypertrophy. *PLoS One* **2012**, *7*, e41545.
(43) Lin, C. C.; Huang, C. Y.; Mong, M. C.; Chan, C. Y.; Yin, M. C. Antiangiogenic potential of three triterpenic acids in human liver cancer cells. *J. Agric. Food Chem.* **2011**, *59*, 755−762.
(44) Wang, X.; Bai, H.; Zhang, X.; Liu, J.; Cao, P.; Liao, N.; Zhang, W.; Wang, Z.; Hai, C. Inhibitory effect of oleanolic acid on hepatocellular carcinoma via ERK-p53-mediated cell cycle arrest and mitochondrial-dependent apoptosis. *Carcinogenesis* **2013**, *34*, 1323−1330.
(45) George, V. C.; Kumar, D. R.; Suresh, P. K.; Kumar, R. A. Apoptosis-induced cell death due to oleanolic acid in HaCaT keratinocyte cells − a proof-of-principle approach for chemopreventive drug development. *Asian Pac. J. Cancer Prev.* **2012**, *13*, 2015−2020.
(46) Rufino-Palomares, E. E.; Reyes-Zurita, F. J.; Garcia-Salguero, L.; Mokhtari, K.; Medina, P. P.; Lupianez, J. A.; Peragon, J. Maslinic acid, a triterpenic anti-tumoural agent, interferes with cytoskeleton protein expression in HT29 human colon-cancer cells. *J. Proteomics* **2013**, *83C*, 15−25.
(47) Lúcio, K. A.; da Graça Rocha, G.; Monção-Ribeiro, L. C.; Fernandes, J.; Takiya, C. M.; Gattass, C. R. Oleanolic acid initiates apoptosis in non-small cell lung cancer cell lines and reduces metastasis of a B16F10 melanoma model in vivo. *PLoS One* **2011**, *6*, e28596.
(48) Shan, J. Z.; Xuan, Y. Y.; Ruan, S. Q.; Sun, M. Proliferation-inhibiting and apoptosis-inducing effects of ursolic acid and oleanolic acid on multi-drug resistance cancer cells in vitro. *Chin. J. Integr. Med.* **2011**, *17*, 607−611.
(49) Li, C.; Yang, Z.; Zhai, C.; Qiu, W.; Li, D.; Yi, Z.; Wang, L.; Tang, J.; Qian, M.; Luo, J.; Liu, M. Maslinic acid potentiates the anti-tumor activity of tumor necrosis factor alpha by inhibiting NF-kappaB signaling pathway. *Mol. Cancer* **2010**, *9*, 73.
(50) Petronelli, A.; Pannitteri, G.; Testa, U. Triterpenoids as new promising anticancer drugs. *Anticancer Drugs* **2009**, *20*, 880−892.
(51) Sogno, I.; Vannini, N.; Lorusso, G.; Cammarota, R.; Noonan, D. M.; Generoso, L.; Sporn, M. B.; Albini, A. Anti-angiogenic activity of a novel class of chemopreventive compounds: oleanic acid terpenoids. *Recent Results Cancer Res.* **2009**, *181*, 209−212.
(52) Leal, A. S.; Wang, R.; Salvador, J. A.; Jing, Y. Synthesis of novel heterocyclic oleanolic acid derivatives with improved antiproliferative activity in solid tumor cells. *Org. Biomol. Chem.* **2013**, *11*, 1726−1738.
(53) Shyu, M. H.; Kao, T. C.; Yen, G. C. Oleanolic acid and ursolic acid induce apoptosis in HuH7 human hepatocellular carcinoma cells through a mitochondrial-dependent pathway and downregulation of XIAP. *J. Agric. Food Chem.* **2010**, *58*, 6110−6118.
(54) Wei, J.; Liu, M.; Liu, H.; Wang, H.; Wang, F.; Zhang, Y.; Han, L.; Lin, X. Oleanolic acid arrests cell cycle and induces apoptosis via ROS-mediated mitochondrial depolarization and lysosomal membrane permeabilization in human pancreatic cancer cells. *J. Appl. Toxicol.* **2013**, *33*, 756−765.
(55) Pratheeshkumar, P.; Kuttan, G. Oleanolic acid induces apoptosis by modulating p53, Bax, Bcl-2 and caspase-3 gene expression and regulates the activation of transcription factors and cytokine profile in B16F. *J. Environ. Pathol. Toxicol. Oncol.* **2011**, *30*, 21−31.
(56) Feng, L.; Au-Yeung, W.; Xu, Y. H.; Wang, S. S.; Zhu, Q.; Xiang, P. Oleanolic acid from *Prunella vulgaris* L. induces SPC-A-1 cell line apoptosis via regulation of Bax, Bad and Bcl-2 expression. *Asian Pac. J. Cancer Prev.* **2011**, *12*, 403−408.
(57) Juan, M. E.; Planas, J. M.; Ruiz-Gutierrez, V.; Daniel, H.; Wenzel, U. Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives, on HT-29 colon cancer cells. *Br. J. Nutr.* **2008**, *100*, 36−43.
(58) Yan, S. L.; Huang, C. Y.; Wu, S. T.; Yin, M. C. Oleanolic acid and ursolic acid induce apoptosis in four human liver cancer cell lines. *Toxicol. In Vitro* **2010**, *24*, 842−848.
(59) Zhou, R.; Zhang, Z.; Zhao, L.; Jia, C.; Xu, S.; Mai, Q.; Lu, M.; Huang, M.; Wang, L.; Wang, X.; Jin, D.; Bai, X. Inhibition of mTOR signaling by oleanolic acid contributes to its anti-tumor activity in osteosarcoma cells. *J. Orthop. Res.* **2011**, *29*, 846−852.
(60) Sanchez-Tena, S.; Reyes-Zurita, F. J.; Diaz-Moralli, S.; Vinardell, M. P.; Reed, M.; Garcia-Garcia, F.; Dopazo, J.; Lupianez, J. A.; Gunther, U.; Cascante, M. Maslinic acid-enriched diet decreases intestinal tumorigenesis in Apc(Min/+) mice through transcriptomic and metabolomic reprogramming. *PLoS One* **2013**, *8*, e59392.
(61) Hsum, Y. W.; Yew, W. T.; Hong, P. L.; Soo, K. K.; Hoon, L. S.; Chieng, Y. C.; Mooi, L. Y. Cancer chemopreventive activity of maslinic acid: suppression of COX-2 expression and inhibition of NF-kappaB and AP-1 activation in Raji cells. *Planta Med.* **2011**, *77*, 152−157.
(62) Park, S. Y.; Nho, C. W.; Kwon, D. Y.; Kang, Y. H.; Lee, K. W.; Park, J. H. Maslinic acid inhibits the metastatic capacity of DU145 human prostate cancer cells: possible mediation via hypoxia-inducible factor-1alpha signalling. *Br. J. Nutr.* **2013**, *109*, 210−222.
(63) Parra, A.; Rivas, F.; Martin-Fonseca, S.; Garcia-Granados, A.; Martinez, A. Maslinic acid derivatives induce significant apoptosis in b16f10 murine melanoma cells. *Eur. J. Med. Chem.* **2011**, *46*, 5991−6001.

(64) Reyes-Zurita, F. J.; Pachon-Pena, G.; Lizarraga, D.; Rufino-Palomares, E. E.; Cascante, M.; Lupianez, J. A. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer* **2011**, *11*, 154.
(65) Wu, D. M.; Zhao, D.; Li, D. Z.; Xu, D. Y.; Chu, W. F.; Wang, X. F. Maslinic acid induces apoptosis in salivary gland adenoid cystic carcinoma cells by Ca^{2+}-evoked p38 signaling pathway. *Naunyn Schmiedebergs Arch. Pharmacol.* **2011**, *383*, 321−330.
(66) Reyes-Zurita, F. J.; Rufino-Palomares, E. E.; Lupianez, J. A.; Cascante, M. Maslinic acid, a natural triterpene from *Olea europaea* L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. *Cancer Lett.* **2009**, *273*, 44−54.
(67) Juan, M. E.; Planas, J. M.; Ruiz-Gutierrez, V.; Daniel, H.; Wenzel, U. Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives, on HT-29 colon cancer cells. *Br. J. Nutr.* **2008**, *100*, 36−43.
(68) Li, C.; Yang, Z.; Zhai, C.; Qiu, W.; Li, D.; Yi, Z.; Wang, L.; Tang, J.; Qian, M.; Luo, J.; Liu, M. Maslinic acid potentiates the anti-tumor activity of tumor necrosis factor alpha by inhibiting NF-kappaB signaling pathway. *Mol. Cancer* **2010**, *9*, 73.
(69) Trumbull, E. R.; Bianchi, E.; Eckert, D. J.; Wiedhopf, R. M.; Cole, J. R. Tumor inhibitory agents from *Vauquelinia corymbosa* (Rosaceae). *J. Pharm. Sci.* **1976**, *65*, 1407−1408.
(70) Es-Saady, D.; Najid, A.; Simon, A.; Denizot, Y.; Chulia, A. J.; Delage, C. Effects of ursolic acid and its analogues on soybean 15-lipoxygenase activity and the proliferation rate of a human gastric tumour cell line. *Mediators Inflamm.* **1994**, *3*, 181−184.
(71) Nishino, H.; Nishino, A.; Takayasu, J.; Hasegawa, T.; Iwashima, A.; Hirabayashi, K.; Iwata, S.; Shibata, S. Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. *Cancer Res.* **1988**, *48*, 5210−5215.
(72) Juan, M. E.; Wenzel, U.; Daniel, H.; Planas, J. M. Erythrodiol, a natural triterpenoid from olives, has antiproliferative and apoptotic activity in HT-29 human adenocarcinoma cells. *Mol. Nutr. Food Res.* **2008**, *52*, 595−599.
(73) Min, B. S.; Kim, Y. H.; Lee, S. M.; Jung, H. J.; Lee, J. S.; Na, M. K.; Lee, C. O.; Lee, J. P.; Bae, K. Cytotoxic triterpenes from *Crataegus pinnatifida*. *Arch. Pharm. Res.* **2000**, *23*, 155−158.
(74) Martin, R.; Ibeas, E.; Carvalho-Tavares, J.; Hernandez, M.; Ruiz-Gutierrez, V.; Nieto, M. L. Natural triterpenic diols promote apoptosis in astrocytoma cells through ROS-mediated mitochondrial depolarization and JNK activation. *PLoS One* **2009**, *4*, e5975.
(75) Martins, A.; Vasas, A.; Schelz, Z.; Viveiros, M.; Molnar, J.; Hohmann, J.; Amaral, L. Constituents of *Carpobrotus edulis* inhibit P-glycoprotein of MDR1-transfected mouse lymphoma cells. *Anticancer Res.* **2010**, *30*, 829−835.
(76) Chen, H. L.; Lin, K. W.; Huang, A. M.; Tu, H. Y.; Wei, B. L.; Hour, T. C.; Yen, M. H.; Pu, Y. S.; Lin, C. N. Terpenoids induce cell cycle arrest and apoptosis from the stems of *Celastrus kusanoi* associated with reactive oxygen species. *J. Agric. Food Chem.* **2010**, *58*, 3808−3812.
(77) Singh, G. B.; Singh, S.; Bani, S.; Gupta, B. D.; Banerjee, S. K. Anti-inflammatory activity of oleanolic acid in rats and mice. *J. Pharm. Pharmacol.* **1992**, *44*, 456−458.
(78) Kapil, A.; Sharma, S. Effect of oleanolic acid on complement in adjuvant- and carrageenan-induced inflammation in rats. *J. Pharm. Pharmacol.* **1995**, *47*, 585−587.
(79) Manez, S.; Recio, M. C.; Giner, R. M.; Rios, J. L. Effect of selected triterpenoids on chronic dermal inflammation. *Eur. J. Pharmacol.* **1997**, *334*, 103−105.
(80) Suh, S. J.; Jin, U. H.; Kim, K. W.; Son, J. K.; Lee, S. H.; Son, K. H.; Chang, H. W.; Lee, Y. C.; Kim, C. H. Triterpenoid saponin, oleanolic acid 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside (OA) from *Aralia elata* inhibits LPS-induced nitric oxide production by down-regulated NF-κB in raw 264.7 cells. *Arch. Biochem. Biophys.* **2007**, *467*, 227−233.
(81) Marquez-Martin, A.; Puerta, R. D. L.; Fernandez-Arche, A.; Ruiz-Gutierrez, V.; Yaqoob, P. Modulation of cytokine secretion by pentacyclic triterpenes from olive pomace oil in human mononuclear cells. *Cytokine* **2006**, *36*, 211−217.
(82) Yang, E. J.; Lee, W.; Ku, S. K.; Song, K. S.; Bae, J. S. Anti-inflammatory activities of oleanolic acid on HMGB1 activated HUVECs. *Food Chem. Toxicol.* **2012**, *50*, 1288−1294.
(83) Lee, W.; Yang, E. J.; Ku, S. K.; Song, K. S.; Bae, J. S. Anti-inflammatory effects of oleanolic acid on LPS-induced inflammation in vitro and in vivo. *Inflammation* **2013**, *36*, 94−102.
(84) Buttke, T. M.; Sandstrom, P. A. Oxidative stress as a mediator of apoptosis. *Immunol. Today* **1994**, *15*, 7−10.
(85) Oita, R. C.; Ferdinando, D.; Wilson, S.; Bunce, C.; Mazzatti, D. J. Visfatin induces oxidative stress in differentiated C2C12 myotubes in an Akt- and MAPK-independent, NFkB-dependent manner. *Pfluegers Arch.* **2010**, *459*, 619−630.
(86) Balanehru, S.; Nagarajan, B. Protective effect of oleanolic acid and ursolic acid against lipid peroxidation. *Biochem. Int.* **1991**, *24*, 981−990.
(87) Montilla, M. P.; Agil, A.; Navarro, M. C.; Jimenez, M. I.; Garcia-Granados, A.; Parra, A.; Cabo, M. M. Antioxidant activity of maslinic acid, a triterpene derivative obtained from *Olea europaea*. *Planta Med.* **2003**, *69*, 472−474.
(88) Perona, J. S.; Arcemis, C.; Ruiz-Gutierrez, V.; Catala, A. Effect of dietary high-oleic-acid oils that are rich in antioxidants on microsomal lipid peroxidation in rats. *J. Agric. Food Chem.* **2005**, *53*, 730−735.
(89) Diaz, A. M.; Abad, M. J.; Fernandez, L.; Recuero, C.; Villaescusa, L.; Silvan, A. M.; Bermejo, P. In vitro anti-inflammatory activity of iridoids and triterpenoid compounds isolated from *Phillyrea latifolia* L. *Biol. Pharm. Bull.* **2000**, *23*, 1307−1313.
(90) Huang, L.; Guan, T.; Qian, Y.; Huang, M.; Tang, X.; Li, Y.; Sun, H. Anti-inflammatory effects of maslinic acid, a natural triterpene, in cultured cortical astrocytes via suppression of nuclear factor-kappa B. *Eur. J. Pharmacol.* **2011**, *672*, 169−174.
(91) Marquez Martin, A.; de la Puerta Vazquez, R.; Fernandez-Arche, A.; Ruiz-Gutierrez, V. Supressive effect of maslinic acid from pomace olive oil on oxidative stress and cytokine production in stimulated murine macrophages. *Free Radical Res.* **2006**, *40*, 295−302.
(92) Yang, Z. G.; Li, H. R.; Wang, L. Y.; Li, Y. H.; Lu, S. G.; Wen, X. F.; Wang, J.; Daikonya, A.; Kitanaka, S. Triterpenoids from *Hippophae rhamnoides* L. and their nitric oxide production-inhibitory and DPPH radical-scavenging activities. *Chem. Pharm. Bull. (Tokyo)* **2007**, *55*, 15−18.
(93) Tsai, S. J.; Yin, M. C. Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells. *J. Food Sci.* **2008**, *73*, H174−H178.
(94) Rong, Z. T.; Gong, X. J.; Sun, H. B.; Li, Y. M.; Ji, H. Protective effects of oleanolic acid on cerebral ischemic damage in vivo and H(2)O(2)-induced injury in vitro. *Pharm. Biol.* **2011**, *49*, 78−85.
(95) Tsai, S. J.; Yin, M. C. Anti-oxidative, anti-glycative and anti-apoptotic effects of oleanolic acid in brain of mice treated by D-galactose. *Eur. J. Pharmacol.* **2012**, *689*, 81−88.
(96) Ovesna, Z.; Kozics, K.; Slamenova, D. Protective effects of ursolic acid and oleanolic acid in leukemic cells. *Mutat. Res.* **2006**, *600*, 131−137.
(97) Hoeschen, R. J. Oxidative stress and cardiovascular disease. *Can. J. Cardiol.* **1997**, *13*, 1021−1025.
(98) Halliwell, B. Oxidative stress and cancer: have we moved forward? *Biochem. J.* **2007**, *401*, 1−11.
(99) Hwang, O. Role of oxidative stress in Parkinson's disease. *Exp. Neurobiol.* **2013**, *22*, 11−17.
(100) Markesbery, W. R. Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biol. Med.* **1997**, *23*, 134−147.
(101) Nunomura, A.; Perry, G.; Aliev, G.; Hirai, K.; Takeda, A.; Balraj, E. K.; Jones, P. K.; Ghanbari, H.; Wataya, T.; Shimohama, S.; Chiba, S.; Atwood, C. S.; Petersen, R. B.; Smith, M. A. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **2001**, *60*, 759−767.
(102) Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272*, 20313−20316.
(103) Jeong, D. W.; Wang, B. J.; Wei, C. M.; Yuan, G. Y.; Bu, F. L.; Guo, W. R.; Lee, S. M.; Han, C. K.; Lee, H. S. Dose-linear pharmacokinetics of

oleanolic acid after intravenous and oral administration in rats. *Biopharm. Drug Dispos.* **2007**, *28*, 51−57.
(104) Song, M.; Hang, T. J.; Wang, Y.; Jiang, L.; Wu, X. L.; Zhang, Z.; Shen, J.; Zhang, Y. Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2006**, *40*, 190−196.
(105) Lozano-Mena, G.; Juan, M. E.; García-Granados, A.; Planas, J. M. Determination of maslinic acid, a pentacyclic triterpene from olives, in rat plasma by high-performance liquid chromatography. *J. Agric. Food Chem.* **2012**, *60*, 10220−10225.
(106) Rada, M.; Ruiz-Gutiérrez, V.; Guinda, Á. Determination of triterpenic acids in human serum by high-performance liquid chromatography: triterpenoid interaction with serum protein. *J. Agric. Food Chem.* **2011**, *59*, 2308−2313.
(107) Romero, C.; García, A.; Medina, E.; Ruiz-Mendez, M. V.; Castro, A. D.; Brenes, M. Triterpenic acids in table olives. *Food Chem.* **2010**, *118*, 670−674.
(108) Guinda, Á.; Rada, M.; Delgado, T.; Gutierrez-Adanez, P.; Castellano, J. M. Pentacyclic triterpenoids from olive fruit and leaf. *J. Agric. Food Chem.* **2010**, *58*, 9685−9691.
(109) Sánchez-Gonzalez, M.; Lozano-Mena, G.; Juan, M. E.; García-Granados, A.; Planas, J. M. Assessment of the safety of maslinic acid, a bioactive compound from *Olea europaea* L. *Mol. Nutr. Food Res.* **2013**, *57*, 339−346.

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

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Uvaol (UV) and erythrodiol (ER) are two triterpenic dialcohols present in the minor fraction of virgin olive oil, in leaves and in the drupe of olives. These triterpenes possess the same chemical structure and differ only 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 effects 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). UV and ER showed different effects in normal and breast cancer cells, whereas both compounds possess the same structure, except for the location of a methyl group. UV protects from damage to DNA in both cell lines, whereas ER enhances damage to DNA in these cell lines. Thus, ER promotes apoptosis and arrests cell cycle in human epithelial breast cells. Hence, both compounds differ in their action in human breast cells apparently by the different location of only a methyl group.

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Introduction

Uvaol (UV) and erythrodiol (ER) are two compounds present in the leaves, fruit of olive tree and in virgin olive oil.[1] These two compounds (with the same molecular formula and weight, *i.e.* $C_{30}H_{50}O_2$ and 442.7168, respectively) possess two hydroxyl groups in remote positions and differ in the location of the methyl group, which is located at carbon 19 for uvaol and at carbon 20 for erythrodiol (Fig. 1). UV and ER are described to possess multiple activities against different kinds of cancer cells.[2–5] However, to date, there is no reference about the role that these two dialcohols play in metastatic breast cancer cells or in human non-tumorigenic breast cells.

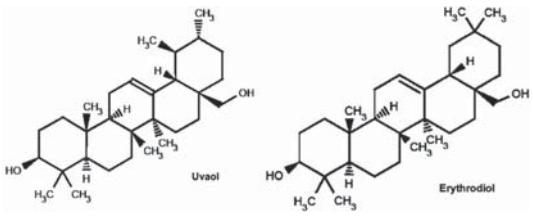

Fig. 1 Chemical structure of uvaol and erythrodiol.

On the other hand, the health benefits of virgin olive oils and the multiple qualities that it possesses in the prevention of several diseases,[6–12] such as breast cancer, are widely known. It is known that, in carcinogenesis, ROS levels are 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 the creation of more H_2O_2 than normal cells. By controlling the ROS levels inside a cancer cell, we could activate apoptosis pathway in the cell, or even prevent carcinogenesis in normal breast 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,[13] which are implicated in intracellular ROS production. UV and ER possess antioxidant activity against lipid peroxidation in rats;[14] therefore, they could be a good option for controlling ROS levels inside the cells.

Taking into account that the control of ROS could play a role in the prevention of breast cancer, it is interesting to know if UV and ER, which are two triterpenes with antioxidant activity, have the capacity to prevent breast cancer development or even have antitumor properties. For this reason, the

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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. However, 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 increasing the number of cells at lower 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).

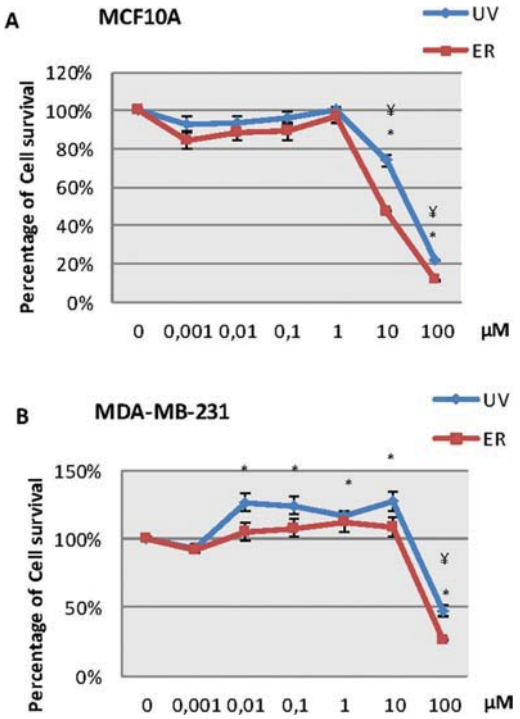

Fig. 2 Cytotoxicity of UV and ER after treatment in a range from 0.001 μM to 100 μM in MCF10A cells (A) and MDA-MB-231 cells (B). Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences at $p < 0.05$ for UV and ER are represented by (*) and (¥), respectively.

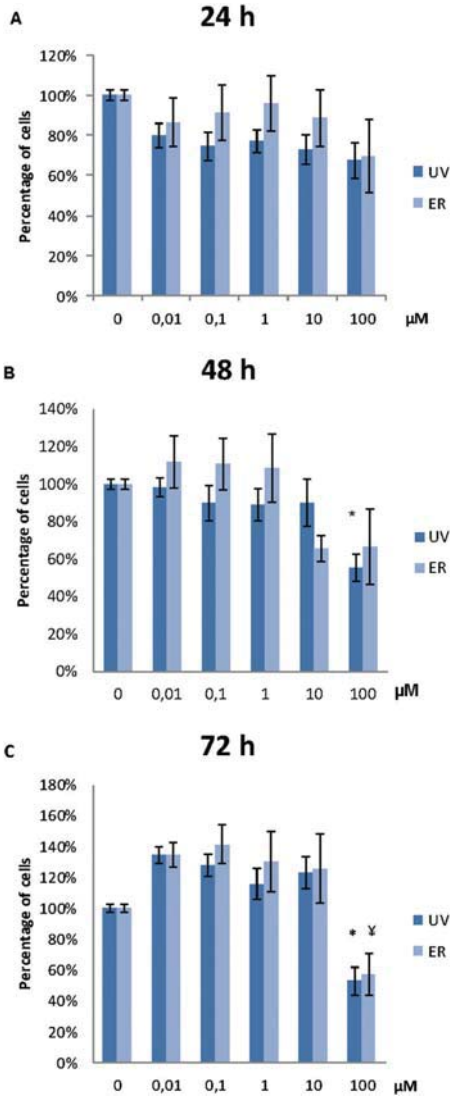

Fig. 3 Proliferation of MCF10A cells after treatments with UV and ER in a range of concentrations from 0.01 μM to 100 μM at 24 h (A), 48 h (B) and 72 h (C). Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at $p < 0.05$.

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

Cell cycle

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 Sub G0/G1 and in G0/G1 phases in

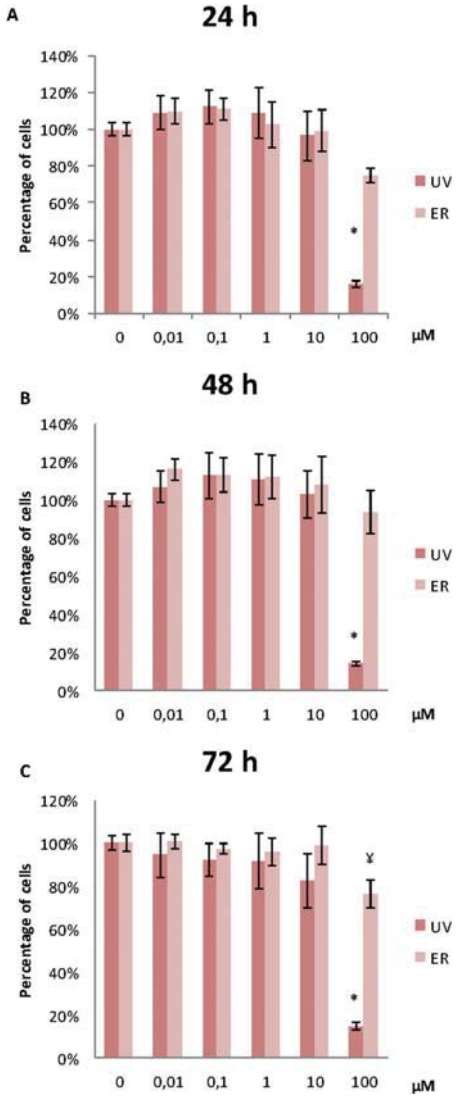

Fig. 4 Proliferation of MDA-MB-231 cells after treatments with UV and ER in a range of concentration from 0.01 μM to 100 μM at 24 h (A), 48 h (B) and 72 h (C). Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at $p < 0.05$.

MCF10A, whereas in MDA-MB-231, no difference was observed respect to the control (data not shown).

Apoptosis analysis

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

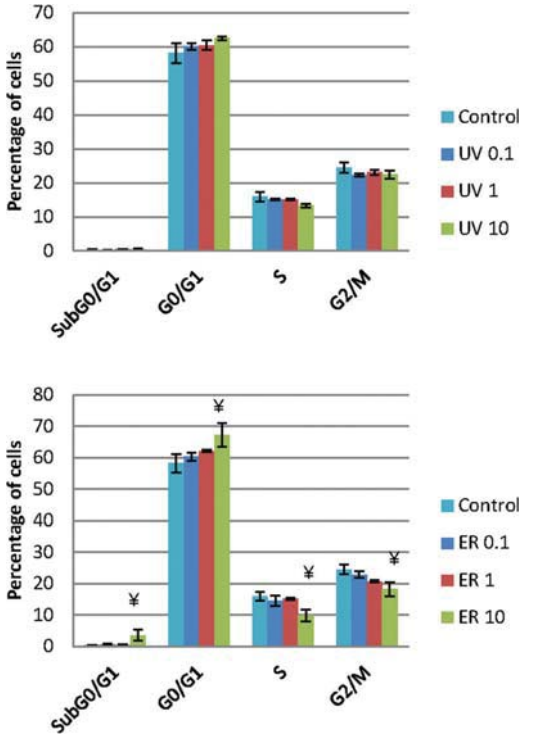

Fig. 5 Cell cycle represented as percentage of cells in the different cell cycle phases of MCF10A cells after treatments with UV and ER at 0.1 μM, 1 μM and 10 μM. Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at $p < 0.05$.

Analysis of intracellular reactive oxygen species

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

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

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

In Fig. 6A and 7A, data are expressed as percentage of relative fluorescence with respect to the untreated cells that are represented as 100%. In Fig. 6B and 7B, data are expressed as percentage of relative fluorescence with respect to positive control (cells only treated with H_2O_2) that are represented as 100% and negative control that are expressed as 1.5% ± 0.2%

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 (*) at $p < 0.05$

	MCF10A			MDA-MB-231		
	Live	Apoptotic	Death	Live	Apoptotic	Death
Camptothecin	62.66 ± 15.35*	26.45 ± 12.55*	10.87 ± 10.07*	57.70 ± 5.66*	16.69 ± 4.57*	25.60 ± 7.15*
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

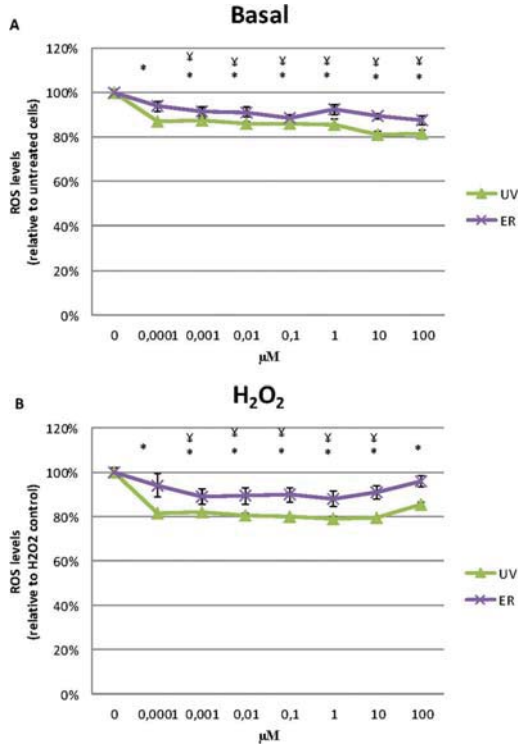

Fig. 6 ROS levels of MCF10A in basal state of the cells (A) and after exposure to H_2O_2 (B) with treatments of UV and ER in a range of concentration between 0.0001 μM and 100 μM after 4 h. Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at $p < 0.05$.

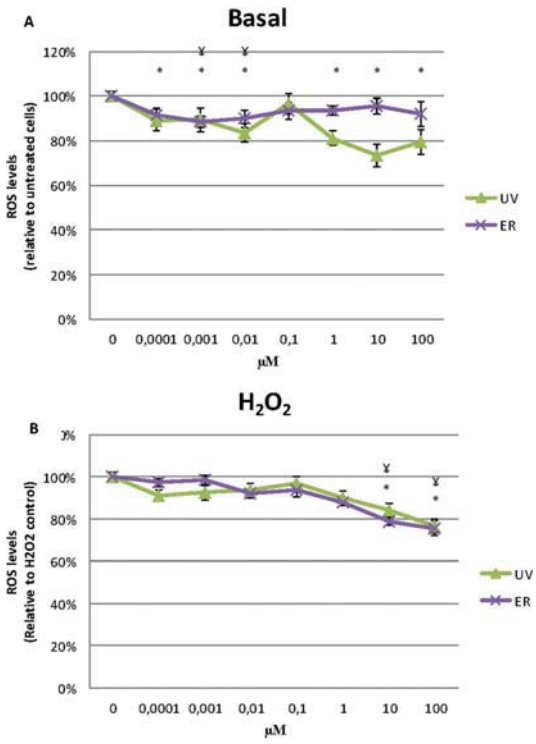

Fig. 7 ROS levels of MDA-MB-231 in basal state of the cells (A) and after exposure to H_2O_2 (B) with treatments of UV and ER in a range of concentration between 0.0001 μM and 100 μM after 4 h. Numerical values are presented as the mean ± SEM of three independent experiments. Statistically significant differences are represented as (*) for UV and (¥) for ER at $p < 0.05$.

for MCF10A and 5% ± 1.4% for MDA-MB-231 (data not shown).

Catalase activity

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

Analysis of comet assay

In MCF10A cells, UV at 0.1 μM reduced the damage to DNA in more than 50% with respect to the control. However, this effect was lost at 1 μM, at which 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 the UV and ER ability to protect against oxidative

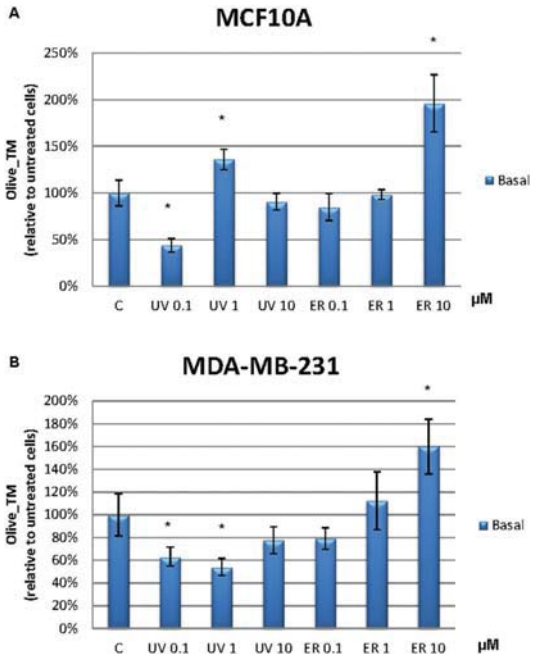

Fig. 8 DNA damage represented as Olive_TM of MCF10A cells (A) and MDA-MB-231 cells (B) in basal state after treatments with UV and ER at 0.1 μM, 1 μM and 10 μM for 4 h. Olive_TM values are represented by mean ± SEM of three independent experiments. Significant differences were determinate relative to the control untreated, which was defined as 100% at $*p < 0.05$.

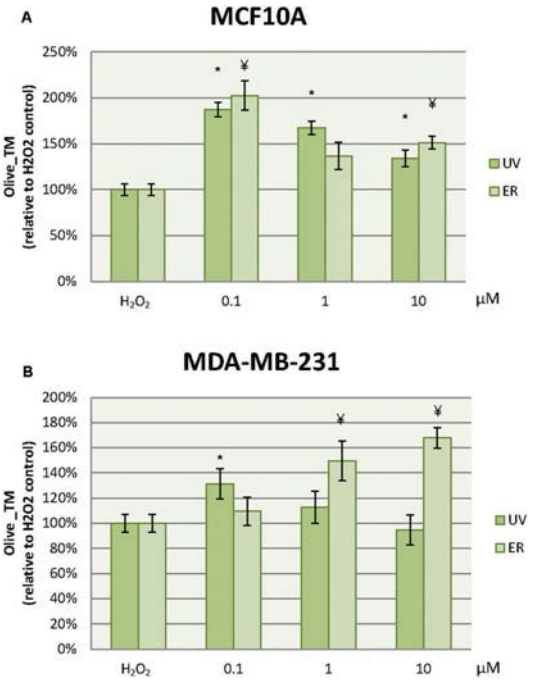

Fig. 9 DNA damage represented as Olive_TM of MCF10A (A) and MDA-MB-231 (B) after treatments with UV and ER at 0.1 μM, 1 μM and 10 μM for 4 h and after an induced oxidative damage with H_2O_2. Olive_TM values are represented by mean ± SEM of three independent experiments. Significant differences were determinate relative to H_2O_2 control, which was defined as 100% at $*p < 0.05$.

DNA damage, cells were exposed to H_2O_2. When oxidative damage was induced in cells, UV and ER increased the damage to DNA with respect to the control in MCF10A (Fig. 9A).

In MDA-MB-231 cells, UV at 0.1 and 1 μM decreased damage to DNA respect to the 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).

In Fig. 8, data are expressed as percentage of Olive_TM with respect to the control untreated (represented as 100%). In Fig. 9, data are expressed as percentage of Olive_TM with respect to the control treated with H_2O_2, which is represented as 100%, and negative control, which is expressed as 14.5% ± 1.8% for MCF10A and 25% ± 3.7% for MDA-MB-231 (data not shown).

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 the difference in the location of the methyl group. These compounds have been described to show different effects related to the control of reactive oxygen species (ROS).[2,5,6,17] Our results showed that the ROS levels were diminished with respect to the control in both human breast cell lines (MCF10A and MDA-MB-231) after treatment with UV and ER. Hence, both compounds are antioxidant in these cells, such as in MCF7.[2] However, when an oxidative stress was induced by H_2O_2, the concentrations ranging between 0.0001 and 1 μM did not exert an antioxidant effect in MDA-MB-231, whereas in MCF10A, they still exerted an antioxidant effect. Thus, it seems that both compounds interfere with the 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 clarified in the 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 promoting apoptosis and necrosis of the malignant cells or could act as a trigger for carcinogenesis by permanent damage of DNA, causing mutations in p53, which is the tumour suppressor gene that is frequently mutated up to 50%.[15]

When an increase of ROS is persistent (for example, in carcinogenesis), it can affect the DNA, proteins or lipids.[18] 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, and it prevented 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 and others). For assessing the effects of ROS on DNA in both cell lines after treatment with UV and ER, the comet assay was prepared. In the cell lines tested, UV acted as an antioxidant protecting the cells against DNA damage (according to the results obtained in oxidative stress measurement), whereas ER promoted damage to DNA. However, when cells were exposed to oxidative damage (H_2O_2 was added), UV and ER at lower concentrations enhanced the damage to DNA in both breast cell lines, and changed their behaviour when we altered the intracellular ROS levels. Both compounds at high concentrations promoted cell death; however, 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, which is induced by H_2O_2. ER could promote stress, and stress could activate c-Jun N-terminal kinase (JNK) pathway, which is known to be involved in apoptosis regulation in several cellular types.[5] In the present study, cells after 4 h of treatment with ER at 10 μM promoted DNA damage. This damage could derivate in apoptosis, in fact, apoptosis after ER treatment has been described by Martín *et al.*[5] in an astrocytoma cell line, 1321N1. This accumulation of ROS is also described in bladder cancer cells (NTUB1), in which ER was capable only with 5 and 10 μM to increase ROS production and arrest the cell cycle in G0/G1 with the corresponding apoptotic cell death at 24 h.[17] Therefore, 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 did 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. The different effect (after enhace ROS levels by H_2O_2) in intracellular oxidative stress is described above; hence, this effect was different if it was a cancer cell or not. Moreover, at 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. Thus, 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 chemicals 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 (1×, 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 from 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 °C in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured using TrypLE Express solution, and those in the exponential growth phase were used for all experiments.

Except for the assays that specified the opposite, cells were treated for 4 h with 0.1 μM, 1 μM and 10 μM of uvaol (UV) and erythrodiol (ER) dissolved in absolute ethanol.

Cytotoxicity assay

Cell survival, which his 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.*[19] with some modifications. Briefly, cells were seeded into 96-well culture plates in a total volume of 100 μL per well (5×10^3 cells per well for MDA-MB-231 and 2.5×10^3 cells per 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 °C with 5% CO_2, and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). Moreover, viability was calculated using the formula:

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

where A is the difference in absorbance between optical density units ($A = OD_{450} - OD_{620}$). All measurements were performed in 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, which is 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 1×10^3 cells per well for MDA-MB-231 and 0.5×10^3 cells per well for MCF10A. After overnight incubation to allow cell attachment, the 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). Moreover, viability was calculated using the formula:

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

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 (1×10^5 cells per well for MDA-MB-231 and 0.5×10^5 cells per well for MCF10A) and incubated overnight to allow attachment of cells. Then, cells were treated with 0.1 μM, 1 μM and 10 μM of UV and ER for 24 h; moreover, cells were harvested with TrypLE Express and washed with 1× PBS (Ca^{2+}/Mg^{2+} free) (300 × g 10 min at 4 °C). Next, 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, and 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 (1×10^5 cells per well for MDA-MB-231 and 0.5×10^5 cells per well for MCF10A) and incubated overnight to allow cell attachment. After exposure of the cells 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 1× PBS (Ca^{2+}/Mg^{2+} free) (300 × g 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), and each experiment was repeated at least three times independently.

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.*[7] with some modifications. Briefly, cells were seeded on a 96-well plate (5×10^3 cells per well for MDA-MB-231 and 2.5×10^3 cells per 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), and 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 $F_{t=30}$ is 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 damages DNA.[20] 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 0.5×10^6 cells per mL for MCF10A and MDA-MB-231, and they were incubated overnight for cells attachment. Next, the medium was replaced by fresh medium with UV and ER. The assay was prepared according to the manufacturer's protocol for the determination of the enzymatic activity of catalase.

Alkaline single-cell gel electrophoresis (comet assay)

Cells were seeded into a 12-well plate (1×10^5 cells per well for MDA-MB-231 and 0.5×10^5 cells per well for MCF10A) and incubated overnight for cells attachment. Then, the cells were treated with UV and ER. Next, cells were scraped and washed twice (300 × g 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free). They were resuspended in 1 mL of cold 1× PBS. In order to evaluate the UV and ER ability to protect against oxidative DNA damage, cells were exposed for 10 min to 50 μM H_2O_2 at 4 °C.

Then, the comet assay was performed according to Warleta *et al.*[7]

Slide scoring and analysis

DNA strand breaks were examined using 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 20× magnifications. Relative fluorescence between head and tail through the olive tail moment (Olive_TM) was used to determine the DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

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

Statistical analysis

Results are displayed as the mean of at least three independent experiments (±SEM), and 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. The 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 differ in their actions in both types 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 prevent damage to DNA in normal breast epithelial cells, while ER could increase oxidative damage in breast cancer cells. Nevertheless, more studies are required to assure these effects in both normal epithelial breast cells and breast cancer cells.

Conflict of interest

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

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Notes and references

1 Y. Allouche, A. Jimenez, M. Uceda, M. P. Aguilera, J. J. Gaforio and G. Beltran, *J. Agric. Food Chem.*, 2009, **57**, 3604–3610.
2 Y. Allouche, F. Warleta, M. Campos, C. Sanchez-Quesada, M. Uceda, G. Beltran and J. J. Gaforio, *J. Agric. Food Chem.*, 2011, **59**, 121–130.
3 D. Es-Saady, A. Najid, A. Simon, Y. Denizot, A. J. Chulia and C. Delage, *Mediators Inflammation*, 1994, **3**, 181–184.
4 M. E. Juan, U. Wenzel, H. Daniel and J. M. Planas, *Mol. Nutr. Food Res.*, 2008, **52**, 595–599.
5 R. Martín, E. Ibeas, J. Carvalho-Tavares, M. Hernandez, V. Ruiz-Gutierrez and M. L. Nieto, *PLoS One*, 2009, **4**, e5975, DOI: 10.1371/journal.pone.0005975.
6 Y. Allouche, G. Beltran, J. J. Gaforio, M. Uceda and M. D. Mesa, *Food Chem. Toxicol.*, 2010, **48**, 2885–2890.
7 F. Warleta, M. Campos, Y. Allouche, C. Sanchez-Quesada, J. Ruiz-Mora, G. Beltran and J. J. Gaforio, *Food Chem. Toxicol.*, 2010, **48**, 1092–1100.
8 F. Warleta, C. S. Quesada, M. Campos, Y. Allouche, G. Beltran and J. J. Gaforio, *Nutrients*, 2011, **3**, 839–857.
9 S. J. Tsai and M. C. Yin, *J. Food Sci.*, 2008, **73**, H174–H178.
10 J. M. Lou-Bonafonte, C. Arnal, M. A. Navarro and J. Osada, *Mol. Nutr. Food Res.*, 2012, **56**, 1043–1057.
11 M. De Lorgeril, S. Renaud, N. Mamelle, P. Salen, J. Martin, I. Monjaud, J. Guidollet, P. Touboul and J. Delaye, *Lancet*, 1994, **343**, 1454–1459.
12 R. Estruch, E. Ros, J. Salas-Salvado, M. I. Covas, D. Corella, F. Aros, E. Gomez-Gracia, V. Ruiz-Gutierrez, M. Fiol, J. Lapetra, R. M. Lamuela-Raventos, L. Serra-Majem, X. Pinto, J. Basora, M. A. Munoz, J. V. Sorli, J. A. Martinez, M. A. Martinez-Gonzalez and the PREDIMED Study Investigators, *N. Engl. J. Med.*, 2013, **368**, 1279–1290.
13 C. Liu, K. Gong, X. Mao and W. Li, *Int. J. Cancer*, 2011, **129**, 1519–1531.
14 J. S. Perona, C. Arcemis, V. Ruiz-Gutierrez and A. Catala, *J. Agric. Food Chem.*, 2005, **53**, 730–735.
15 S. Saeidnia and M. Abdollahi, *Toxicol. Appl. Pharmacol.*, 2013, **271**, 49–63.
16 C. Sanchez-Quesada, A. Lopez-Biedma, F. Warleta, M. Campos, G. Beltran and J. J. Gaforio, *J. Agric. Food Chem.*, 2013, **61**, 12173–12182.
17 H. L. Chen, K. W. Lin, A. M. Huang, H. Y. Tu, B. L. Wei, T. C. Hour, M. H. Yen, Y. S. Pu and C. N. Lin, *J. Agric. Food Chem.*, 2010, **58**, 3808–3812.
18 W. Lopaczynski and S. H. Zeisel, *Nutr. Res.*, 2001, **21**, 295–307.
19 D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff and M. R. Boyd, *Cancer Res.*, 1988, **48**, 4827–4833.
20 D. H. Lee, B. S. Lim, Y. K. Lee and H. C. Yang, *Cell Biol. Toxicol.*, 2006, **22**, 39–46.

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Research Article

Maslinic Acid Enhances Signals for the Recruitment of Macrophages and Their Differentiation to M1 State

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The inflammatory process is involved in the genesis and evolution of different diseases like obesity, cardiovascular disease, and cancer. Macrophages play a central role in inflammation. In addition, 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 macrophage response which represents an important defense mechanism against cancer. Our results indicated that maslinic acid modulated the inflammatory response by enhancing the production of IL-8, IL-1α, and IL-1β; it promoted M1 response through the synthesis of IFN-γ; and finally it did not modify significantly the levels of NF$\kappa\beta$ or NO. Overall, our results showed that maslinic acid could prevent chronic inflammation, which represents a crucial step in the development of some cancers.

1. Introduction

It is well known that patients with chronic inflammation 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* studies showed signals of inflammation in multiple pathways related to cancer development [2]. Since the evidences showed that immune cells are able to regulate almost every stage of cancer development, it would be interesting to explore biological mechanisms that could have the potential to modulate the immune response in order to reduce risks.

Macrophages play a central role in the development and maintenance of the inflammatory response. Furthermore, macrophages represent the predominant cellular type of the innate immune response found within tumors and are known as tumor-associated macrophages (TAMs). For example, breast cancer is characterized by having a large population of TAMs. Additionally, TAMs release factors to decrease the local proinflammatory 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 proinflammatory cytokines, anti-inflammatory cytokines, or proteins related with the whole inflammation process such as nuclear factor kappa beta (NF-$\kappa\beta$), which in turn can trigger the synthesis of the proinflammatory cytokine IL-1β. The activation of NF-$\kappa\beta$ into inflammatory response could be induced by other proinflammatory molecules like nitric oxide or by feedback of proinflammatory cytokines (IL-1, TNF-α) [4]. TAMs come from monocytic precursors and undergo specific differentiation depending on local cues in the tissue.

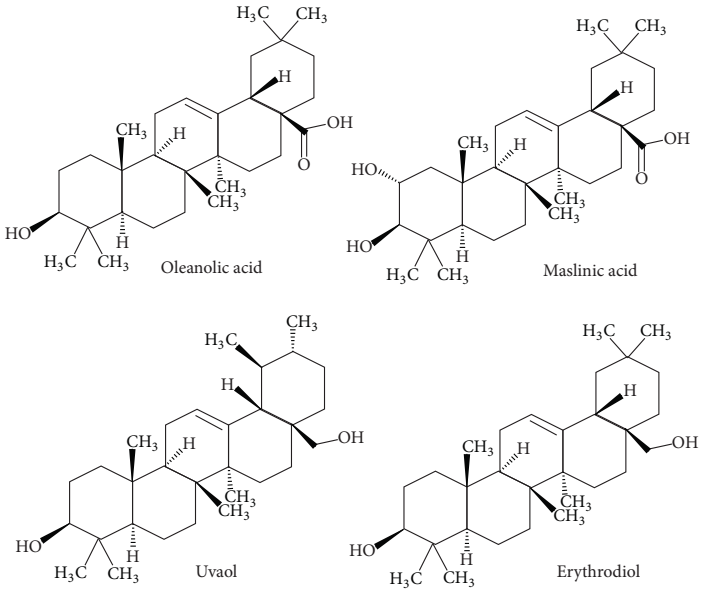

Figure 1: Chemical structure of oleanolic acid, maslinic acid, uvaol, and erythrodiol triterpenes.

Macrophages can be differentiated to M1 macrophages or M2 macrophages. M1 macrophages activate type 1 helper T cells (Th1), they are able to kill pathogens, and they are tumoricidal. On the other hand, M2 macrophages activate type 2 helper T cells (Th2), they are involved in wound healing where they downregulate the inflammatory reactions and promote angiogenesis, and they have a weak tumoricidal capability [5]. TAMs are often abundantly present in malignant tumors and 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 to modulate the inflammatory response in carcinogenesis; it could act against cancer by promoting a Th1 cytotoxic response.

On the other hand, oleanolic acid (OA), maslinic acid (MAS), uvaol (UV), and erythrodiol (ER) are the main pentacyclic triterpenes (Figure 1) 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 [9, 10]. It is believed that virgin olive oil is responsible for these beneficial effects, among other things, because of its anticarcinogenic properties and protection against DNA damages [11–15]. The main triterpenes of virgin olive oil have been described to possess cardioprotective activities [16, 17], antitumor properties [7, 15], and anti-inflammatory activity [18–22]. These triterpenes are synthesized in the leaves and drupe of olive tree, and they are formed from the 2,3-oxidosqualene skeleton. The oleanolic and maslinic acids derive from the oleanane structure, while uvaol derives from ursane structure [23]. The difference between maslinic acid and oleanolic acid is an additional OH group in maslinic acid structure (carbon 2) (Figure 1).

There are no reports on the effect of triterpenes on M1 macrophage response. The present study was designed to investigate the effect of triterpenes found in virgin olive oil on M1 macrophage response.

2. Materials and Methods

2.1. 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%, resp.) 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; nonessential amino acids mixture 100× (NEAA); lipopolysaccharides from *E. 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-methylphenazinium 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 (maximum 0,005%), magnesium chloride (50% $MgCl_2$ powder QP) ($MgCl_2$), and ethanol absolute were 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). NF$\kappa\beta$ 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).

2.2. 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). Cells were maintained at 37°C in a humidified atmosphere with 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 subcultured at least twice per week, 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 (1×10^6 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) to 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.

2.3. Cytotoxicity Assay. THP-1 cells survival, measured as the cellular growth of treated cells versus untreated cells, was carried out using an XTT-based assay according to Warleta et al. [13]. 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, fresh medium was added with triterpenes in a range of concentrations from 0,001 μM to 100 μM of OA, MAS, UV, or ER for 24 h. Thereafter, cells were incubated with XTT in phenol-red-free RPMI medium 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 following formula:

$$\%\ \text{viable cells} = \left[\frac{(A\ \text{treated cells})}{(A\ \text{control})}\right] \times 100, \quad (1)$$

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.

2.4. RayBio Human Cytokine Antibody Array in M1 State THP-1 Macrophages. Differentiated THP-1 cells were stimulated with LPS (1 μg/mL) for 24 h. After that, cells were treated with triterpenes. A negative control (cells undifferentiated and untreated) was also tested. Then, supernatants were isolated and processed according to manufacturer instructions. Arrays membranes were directly detected using a chemiluminescence imaging system (FluorChem E System, ProteinSimple) to achieve production levels of the following cytokines/proteins: eotaxins, 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), and metallopeptidase inhibitor 2 (TIMP-2).

Data were analyzed with the RayBio Human Inflammation Antibody Array 1 Analysis Tool (Catalogue number 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 chemiluminescence 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).

2.5. Flow Cytometry for NF$\kappa\beta$ Detection in M1 State THP-1 Macrophages. After stimulation of differentiated THP-1 cells with LPS (1 μg/mL), cells were treated with OA, MAS, UV, and ER at 1, 10, and 100 μM. Cells were harvested with TrypLE Express and centrifugated at 300 ×g at 4°C for 10 min. The supernatant was discarded and 150 μL of methanol was added. Cells were incubated 10 min at −20°C and washed with PBS. Then, 1 mL of PIPES buffer (PIPES 10 mM, NaCl 0,1 M, $MgCl_2$ 2 mM, and 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-NF$\kappa\beta$ antibody buffer (1 μg/100 μL) on darkness at RT for 30 min. Later, cells were washed and analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). NF-$\kappa\beta$ production was calculated using the FlowJo program (v5.7.2). Each experiment was repeated at least three independent times. Data are represented as percentage of production of NF$\kappa\beta$ with respect to control, which was set as 100%.

2.6. NO Production in M1 Type THP-1 Macrophages. Nitric oxide (NO) production was measured according to F. Amano with some modifications [24]. Differentiated THP-1 cells (5×10^5 cells/mL) were seeded on a 12-well plate and treated with OA, MAS, UV, or ER at 0.1, 1, and 10 μM for 3 h. Then, LPS (1 μg/mL) and L-arginine (L-Arg) at 10 mM were added to cells and incubated for 24 h. Supernatants were

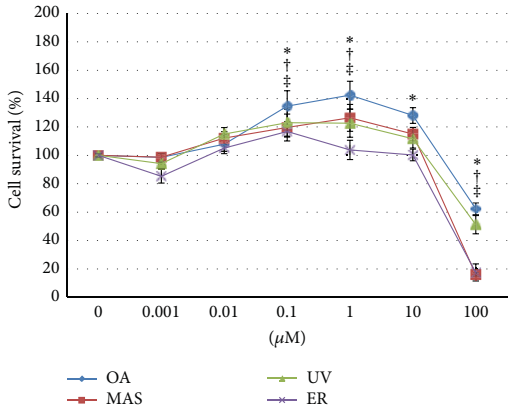

Figure 2: Effects of triterpenes on THP-1 macrophages survival. Cells were differentiated and treated with 0.001-0.01-0.1-1-10-100 μM of oleanolic acid (OA*), maslinic acid (MAS†), uvaol (UV‡), and erythrodiol (ER$^{\Delta}$) at 24 h. $^{(*)(\dagger)(\ddagger)(\Delta)}$Statistically significant differences compared with cells untreated ($P < 0.05$).

collected and incubated with ethanol absolute 30 min at −20°C. Supernatants were centrifuged at 10.000 ×g at 4°C for 10 min and they were aliquoted. Production of NO was measured by a NO analyzer (NOA 280i de SIEVERS, GE Water and Process Technologies, Pennsylvania, USA). Data are expressed as the percentage of NO detection relative to untreated control, which was set as 100%.

2.7. 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 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., Warrenton, VA).

3. Results

3.1. 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 low concentrations, they did not show cytotoxic effects (Figure 2).

3.2. RayBio Human Cytokine Antibody Array. Production of inflammation-related proteins was measured on THP-1 macrophages cells stimulated with LPS (1 μg/mL) for differentiation of M1 phenotype. All the inflammation-related proteins showed significant differences in LPS stimulated cells with respect to untreated cells (Figures 3(a) and 4(a)).

3.2.1. M1/M2 Polarization Related Cytokines. After triterpenic treatments we observed that IFN-γ level, which leads to M1 polarization, was increased with respect to control at MAS 1 μM, 10 μM and ER 1 μM (Figures 3(b) and 3(c)). For the rest of compounds, IFN-γ production levels were similar to control (Figures 3(d) and 3(e)). However, IL-4, which leads to M2 polarization, decreased levels after MAS 1 μM treatment and is absent after MAS 10 μM, ER 1 μM, and 10 μM treatment (Figures 3(b) and 3(c)). IL-10 did not show any significant differences with respect to control. In the other triterpenes tested there were not differences with respect to control (Figures 3(d) and 3(e)). At the concentration of 100 μM, most of the compounds have strong differences with respect to the control, but it might be due to the cytotoxic effects that they exerted at elevated concentrations.

3.2.2. Macrophages Recruitment-Related Cytokines and Proinflammatory Cytokines. Cytokines related with macrophages recruitment such as IL-8, IL-1 alpha, and IL-1 beta appeared increased in macrophages after treatment of MAS at 10 μM. The production of IL-6 cytokine increased at the same concentration (Figure 4(b)). For the rest of compounds only the IL-8 cytokine production was increased at UV 10 μM and IL-1 alpha at ER 10 μM. The IL-6 cytokine levels were increased in all the treatments at 10 μM and at ER 1 μM (Figures 4(c), 4(d), and 4(e)).

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

3.3. Effects on NF-κβ Production. Detection of NF-κβ (p65) was performed by flow cytometry in 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 statistically significant differences between control and samples (Table 1).

3.4. 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 μM, 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 with respect to unstimulated control (data not shown).

4. Discussion

The THP-1 cell line has a closed gene expression to primary macrophages, derived from peripheral blood mononuclear cells, in contrast to other monocytes cell lines like U937 [25]. Furthermore, a PMA differentiation of THP-1 cells drives cells to a differentiated macrophage phenotype that seems very nearby to monocyte-derived human macrophages [26]. Analysis of primary macrophages in culture will always provide more truthful information about inflammation response

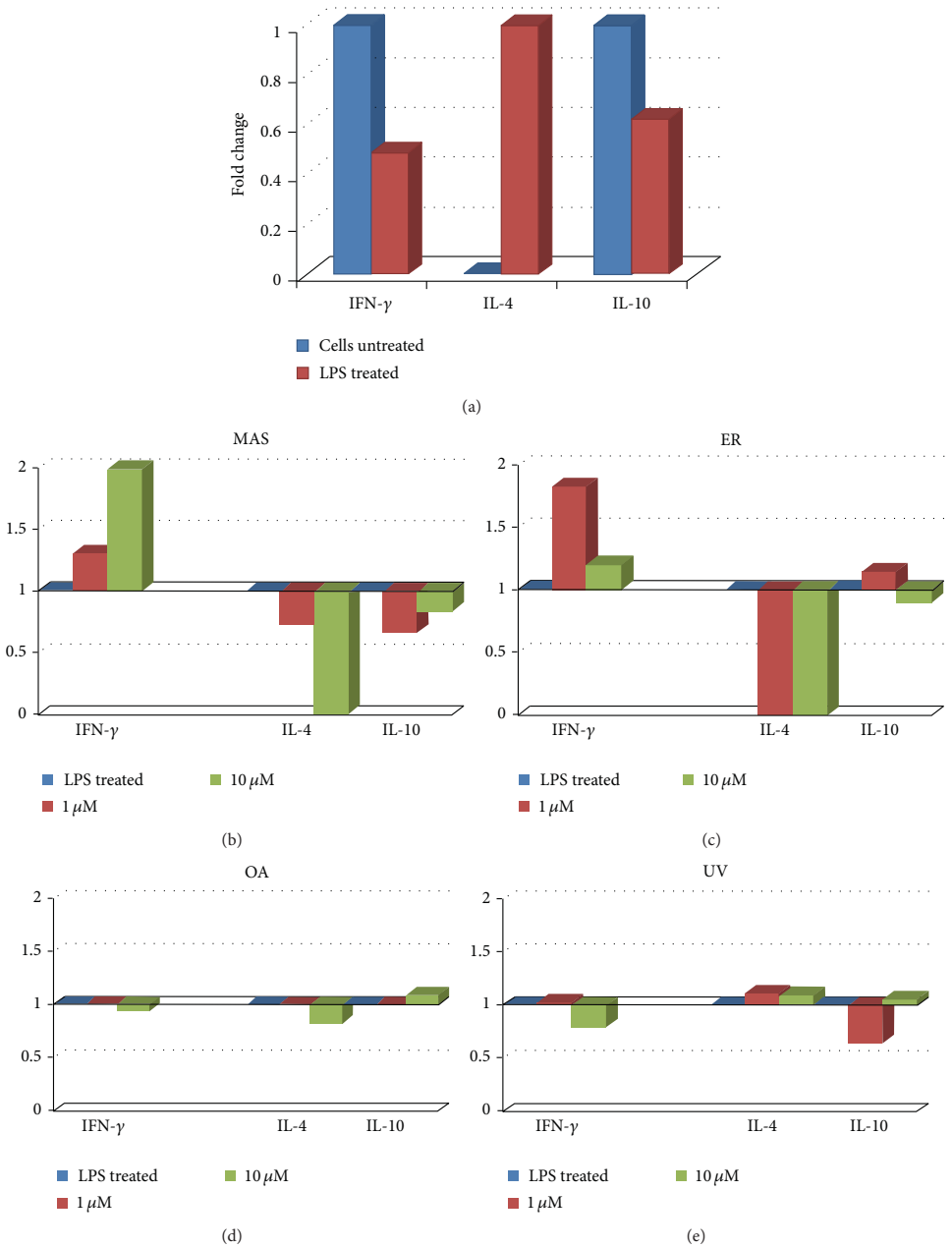

Figure 3: M1 polarization related cytokines production versus M2 polarization related cytokines production in cell unstimulated and stimulated with LPS (a) and in M1 polarized THP-1 macrophages after treatment with MAS (b), ER (c), OA (d), and UV (e) at 1, 10, and 100 μM. Results are expressed as the fold change in RI (relative intensity) related to stimulated control which was set as 1.

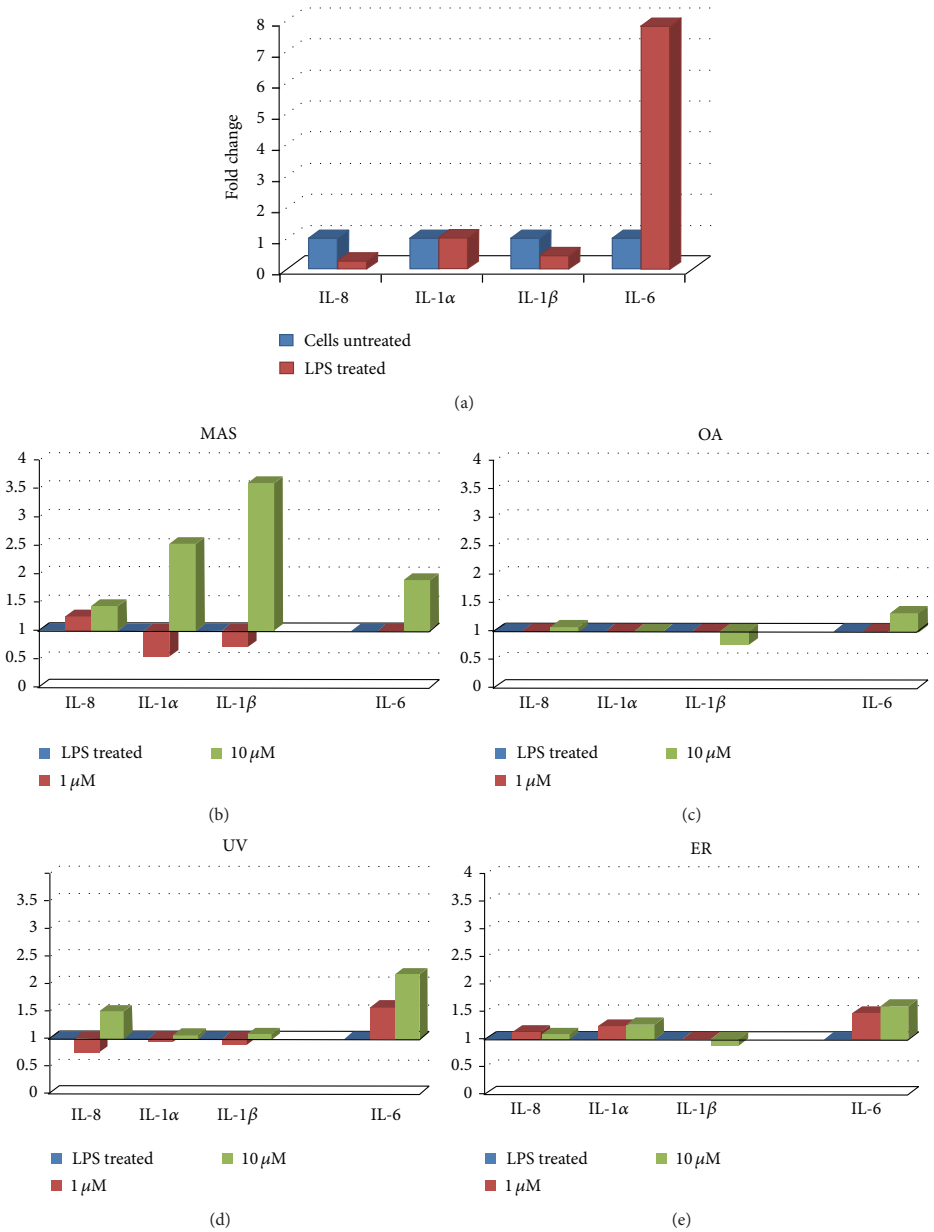

Figure 4: Production of cytokines responsible of macrophage recruitment produced in cell unstimulated and stimulated with LPS (a) and in M1 polarized THP-1 macrophages treated with MAS (b), OA (c), UV (d), and ER (e) at 1, 10, and 100 μM.

than cellular models, 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 treatment with food compounds [27]. Moreover, recently the consequent polarization to M1 phenotype that LPS promotes in THP-1 macrophages has been described [28]. Thus, THP-1

Table 1: NF$\kappa\beta$ 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 the percentage of NF$\kappa\beta$ production with respect to cells LPS treated, which was set as 100%. Standard error means (SEM) represented as percentage. Not statistical differences found at $P < 0.05$.

Treatment	Concentration	Mean	SEM
LPS treated		100	±66,05
OA	1 μM	132	±12,06
	10 μM	74	±17,15
	100 μM	84	±15,74
MAS	1 μM	96	±13,26
	10 μM	101	±17,16
	100 μM	116	±30,50
UV	1 μM	112	±19,54
	10 μM	121	±29,72
	100 μM	102	±23,86
ER	1 μM	110	±38,91
	10 μM	101	±28,62
	100 μM	99	±23,88

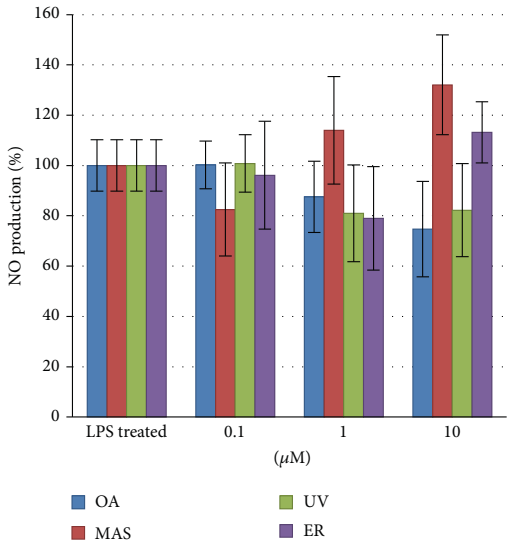

Figure 5: NO production of M1 polarized THP-1 macrophages treated with 0.1, 1, and 10 μM of OA, MAS, UV, and ER. Data are expressed relative to LPS treated cells, which was established as 100%. Not statistical differences were found.

macrophages are the best option for study *in vitro* effects of certain compounds in macrophages differentiated to M1 phenotype.

According to this, THP-1 cells were used to study *in vitro* effects of OA, MA, UV, and ER on M1 macrophages. THP-1 macrophages were differentiated into the M1 stage by LPS treatment.

Macrophages constitute an extremely heterogeneous population, which polarize into distinct macrophages types, mainly identified as M1 (or classically activated) and M2 (or alternatively activated) [29]. Previously, our group described two phenotypically and functionally different populations among splenic macrophages in response to *C. albicans* infection. One of them (M2 phenotype) expressed high levels of major histocompatibility complex (MHC) class II surface expression and is poorly phagocytic. The other one (M1 phenotype) expressed low levels of MHC class II molecules and is highly phagocytic [30]. We suggested 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].

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. These macrophages increase angiogenesis, tumor cell extravasation, and growth; they suppress activation of dendritic cells, cytotoxic T lymphocytes, and natural killers [31, 32].

M1 macrophages appear to have a proangiogenic function early in tumorigenesis [33], when the tumor needs blood vessels formation to grow; this fact 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 in the inflammatory process. 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 downregulating M1-mediated functions. It seems that tumor cells are able to produce several signals that polarize monocytes to M2. This preferential polarization is the result of absence of M1-orienting signals, such as INF-γ or bacterial components in the tumor [29].

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

Our results show that MAS and ER at low concentration increased the production of INF-γ in M1 polarized THP-1 macrophages. By this way, M1 macrophages could mediate and control their own response differentiating monocytes to M1 instead of M2 in carcinogenesis. This increase of INF-γ production would be a proinflammatory signal for monocytes in inflammation locations, differentiating these monocytes to M1 phenotype (Figure 6), making more efficient the recognition of neoplastic cells, and mediating an effective Th1 cells response.

On the other hand, M2 macrophages can switch to M1 at the site of the tumor by INF-γ induction and receptor-mediated activation signals to promote tumor regression [6]. With MAS and ER, it could be M1 polarized macrophages

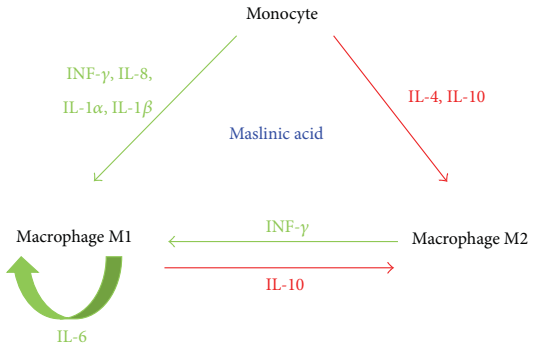

Figure 6: Role of maslinic acid in differentiation and recruitment of macrophages. Cytokine produced after MA treatment in M1 macrophages is represented in green and cytokine inhibited after MA treatment in M1 macrophages is represented in red.

theirselves, which could hypothetically reprogram the M2 macrophages that could show up at the sites of tumor formation, to M1 state.

Indeed, MAS at 10 μM and UV at 10 μM showed levels of IL-8 production slightly higher than the control (Figures 4(b) and 4(d)). Although IL-8 production was not statistically significant, IL-1α and IL-1β were dramatically increased. It is known that the role of IL-8 cytokine on the monocyte recruitment as well as CXCL12 chemokine, whose precursors are IL-1α and IL-1β, interestingly increased in MAS at 10 μM (Figure 4(b)).

Apart from fortifying the proinflammatory response by activating monocytes to M1 and preventing the M2 polarization of monocytes at sites of inflammation, MA appears to promote the recruitment of more cells that could support the immune response on the inflammation location.

Furthermore, some authors describe that NF-$\kappa\beta$ promotes the presence of immunosuppressive M2 phenotype [34]. In order to assess the possible role of triterpenes in promoting production of NF-$\kappa\beta$, we studied its production by the M1 phenotype THP-1 macrophages. It is important to note that a high increase of NF-$\kappa\beta$ expression may lead to an aggravated inflammatory response that could guide to a consequent chronic inflammation [34]. In our study, levels of NF-$\kappa\beta$ were not statically significant with respect to control untreated, 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 [35]. We studied the levels of NO after treatment with triterpenes and there was no statistically significant change in their production compared to the control. These results agree with those of Márquez-Martín et al., who described an inhibition of NO production in peritoneal murine macrophages upon exposure to MA treatment [36]. At 0.1 μM we notify the reduction of NO after MA treatment in M1 macrophages.

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

5. Conclusion

Maslinic acid possesses two principal actions on M1 macrophages: first, it enhanced recruitment of macrophages by production of IL-8, IL-1α, and IL-1β; and second, it promoted M1 response through the synthesis of IFN-γ.

Further studies are needed for assessing the action of these macrophages treated with triterpenes in carcinogenesis. However, maslinic acid could be a useful natural compound to modulate inflammation response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] H. David, "Rudolf Virchow and modern aspects of tumor pathology," *Pathology—Research and Practice*, vol. 183, no. 3, pp. 356–364, 1988.

[2] S. Demaria, E. Pikarsky, M. Karin et al., "Cancer and inflammation: promise for biologic therapy," *Journal of Immunotherapy*, vol. 33, no. 4, pp. 335–351, 2010.

[3] D. Laoui, K. Movahedi, E. van Overmeire et al., "Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions," *International Journal of Developmental Biology*, vol. 55, no. 7–9, pp. 861–867, 2011.

[4] B. B. Aggarwal, S. Shishodia, S. K. Sandur, M. K. Pandey, and G. Sethi, "Inflammation and cancer: how hot is the link?" *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1605–1621, 2006.

[5] S. K. Biswas and A. Mantovani, "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm," *Nature Immunology*, vol. 11, no. 10, pp. 889–896, 2010.

[6] M. Heusinkveld and S. H. van der Burg, "Identification and manipulation of tumor associated macrophages in human cancers," *Journal of Translational Medicine*, vol. 9, no. 1, article 216, 2011.

[7] Y. Allouche, F. Warleta, M. Campos et al., "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," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 1, pp. 121–130, 2011.

[8] F. Perez-Jimenez, G. A. de Cienfuegos, L. Badimon et al., "International conference on the healthy effect of virgin olive

oil," *European Journal of Clinical Investigation*, vol. 35, no. 7, pp. 421–424, 2005.

[9] A. Villarini, P. Pasanisi, A. Traina et al., "Lifestyle and breast cancer recurrences: the DIANA-5 trial," *Tumori*, vol. 98, no. 1, pp. 1–18, 2012.

[10] N. di Daniele, L. Petramala, L. di Renzo et al., "Body composition changes and cardiometabolic benefits of a balanced Italian Mediterranean diet in obese patients with metabolic syndrome," *Acta Diabetologica*, vol. 50, no. 3, pp. 409–416, 2013.

[11] R. Colomer and J. A. Menéndez, "Mediterranean diet, olive oil and cancer," *Clinical and Translational Oncology*, vol. 8, no. 1, pp. 15–21, 2006.

[12] R. W. Owen, R. Haubner, G. Würtele, W. E. Hull, B. Spiegelhalder, and H. Bartsch, "Olives and olive oil in cancer prevention," *European Journal of Cancer Prevention*, vol. 13, no. 4, pp. 319–326, 2004.

[13] F. Warleta, M. Campos, Y. Allouche et al., "Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells," *Food and Chemical Toxicology*, vol. 48, no. 4, pp. 1092–1100, 2010.

[14] F. Warleta, C. S. Quesada, M. Campos, Y. Allouche, G. Beltrán, and J. J. Gaforio, "Hydroxytyrosol protects against oxidative DNA damage in human breast cells," *Nutrients*, vol. 3, no. 10, pp. 839–857, 2011.

[15] C. Sánchez-Quesada, A. López-Biedma, and J. J. Gaforio, "The differential localization of a methyl group confers a different anti-breast cancer activity to two triterpenes present in olives," *Food & Function*, vol. 6, no. 1, pp. 248–255, 2015.

[16] S. Senthil, M. Sridevi, and K. V. Pugalendi, "Cardioprotective effect of oleanolic acid on isoproterenol-induced myocardial ischemia in rats," *Toxicologic Pathology*, vol. 35, no. 3, pp. 418–423, 2007.

[17] Y. Allouche, G. Beltrán, J. J. Gaforio, M. Uceda, and M. D. Mesa, "Antioxidant and antiatherogenic activities of pentacyclic triterpenic diols and acids," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2885–2890, 2010.

[18] R. Martín, M. Hernández, C. Córdova, and M. L. Nieto, "Natural triterpenes modulate immune-inflammatory markers of experimental autoimmune encephalomyelitis: therapeutic implications for multiple sclerosis," *British Journal of Pharmacology*, vol. 166, no. 5, pp. 1708–1723, 2012.

[19] V. S. Graham, C. Lawson, C. P. D. Wheeler-Jones, J. S. Perona, V. Ruiz-Gutierrez, and K. M. Botham, "Triacylglycerol-rich lipoproteins derived from healthy donors fed different olive oils modulate cytokine secretion and cyclooxygenase-2 expression in macrophages: the potential role of oleanolic acid," *European Journal of Nutrition*, vol. 51, no. 3, pp. 301–309, 2012.

[20] C. Li, Z. Yang, C. Zhai et al., "Maslinic acid potentiates the anti-tumor activity of tumor necrosis factor alpha by inhibiting NF-kappaB signaling pathway," *Molecular Cancer*, vol. 9, article 73, 2010.

[21] C. Sánchez-Quesada, A. López-Biedma, F. Warleta, M. Campos, G. Beltrán, and J. J. Gaforio, "Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of *Olea europaea*," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 50, pp. 12173–12182, 2013.

[22] V. R. Yadav, S. Prasad, B. Sung, R. Kannappan, and B. B. Aggarwal, "Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer," *Toxins*, vol. 2, no. 10, pp. 2428–2466, 2010.

[23] N. Stiti, S. Triki, and M.-A. Hartmann, "Formation of triterpenoids throughout *Olea europaea* fruit ontogeny," *Lipids*, vol. 42, no. 1, pp. 55–67, 2007.

[24] F. Amano and T. Noda, "Improved detection of nitric oxide radical (NO) production in an activated macrophage culture with a radical scavenger, carboxy PTIO and griess reagent," *FEBS Letters*, vol. 368, no. 3, pp. 425–428, 1995.

[25] O. Sharif, V. N. Bolshakov, S. Raines, P. Newham, and N. D. Perkins, "Transcriptional profiling of the LPS induced NF-κB response in macrophages," *BMC Immunology*, vol. 8, article 1, 2007.

[26] M. Daigneault, J. A. Preston, H. M. Marriott, M. K. B. Whyte, and D. H. Dockrell, "The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages," *PLoS ONE*, vol. 5, no. 1, Article ID e8668, 2010.

[27] W. Chanput, J. Mes, R. A. M. Vreeburg, H. F. J. Savelkoul, and H. J. Wichers, "Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds," *Food and Function*, vol. 1, no. 3, pp. 254–261, 2010.

[28] W. Chanput, J. J. Mes, H. F. J. Savelkoul, and H. J. Wichers, "Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds," *Food & Function*, vol. 4, no. 2, pp. 266–276, 2013.

[29] G. Solinas, G. Germano, A. Mantovani, and P. Allavena, "Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation," *Journal of Leukocyte Biology*, vol. 86, no. 5, pp. 1065–1073, 2009.

[30] J. J. Gaforio, E. Ortega, I. Algarra, M. J. Serrano, and G. Alvarez de Cienfuegos, "Nk cells mediate increase of phagocytic activity but not of proinflammatory cytokine (interleukin-6 [IL-6], tumor necrosis factor alpha, and IL-12) production elicited in splenic macrophages by tilorone treatment of mice during acute systemic candidiasis," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 6, pp. 1282–1294, 2002.

[31] A. Mantovani and A. Sica, "Macrophages, innate immunity and cancer: balance, tolerance, and diversity," *Current Opinion in Immunology*, vol. 22, no. 2, pp. 231–237, 2010.

[32] B.-Z. Qian and J. W. Pollard, "Macrophage diversity enhances tumor progression and metastasis," *Cell*, vol. 141, no. 1, pp. 39–51, 2010.

[33] S. Samineni, Z. Zhang, and J. E. Shively, "Carcinoembryonic antigen-related cell adhesion molecule 1 negatively regulates granulocyte colony-stimulating factor production by breast tumor-associated macrophages that mediate tumor angiogenesis," *International Journal of Cancer*, vol. 133, no. 2, pp. 394–407, 2013.

[34] L. Jackson and B. M. Evers, "Chronic inflammation and pathogenesis of GI and pancreatic cancers," *Cancer Treatment and Research*, vol. 130, pp. 39–65, 2006.

[35] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[36] A. Márquez Martín, R. de la Puerta Vázquez, A. Fernández-Arche, and V. Ruiz-Gutiérrez, "Supressive effect of maslinic acid from pomace olive oil on oxidative stress and cytokine production in stimulated murine macrophages," *Free Radical Research*, vol. 40, no. 3, pp. 295–302, 2006.

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Article

Oleanolic Acid, a Compound Present in Grapes and Olives, Protects against Genotoxicity in Human Mammary Epithelial Cells

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Abstract: Oleanolic acid (AO) and maslinic acid (MA) are constituents of the skins of different fruits, including olives and white or red grapes. Although both compounds are known to have beneficial properties against different types of cancers, thus far, there are no studies about their chemopreventive effects in human breast cancer. Thus, we sought to elucidate whether both compounds possess chemopreventive activity. Two cell lines of human breast cancer cells and one noncancerous human mammary epithelial cells were used to determine the effects of OA and MA. The results showed that OA inhibited the proliferation and increased the oxidative stress of highly invasive cells. Additionally, OA decreased oxidative stress and oxidative damage to the DNA in human mammary epithelial cells. These results suggest that OA could act as a chemopreventive agent in human breast cancer and could inhibit the proliferation of highly invasive breast cancer cells.

Keywords: virgin olive oil; wine; maslinic acid; MCF7; MDA-MB-231; MCF10A; chemopreventive; antitumoral

1. Introduction

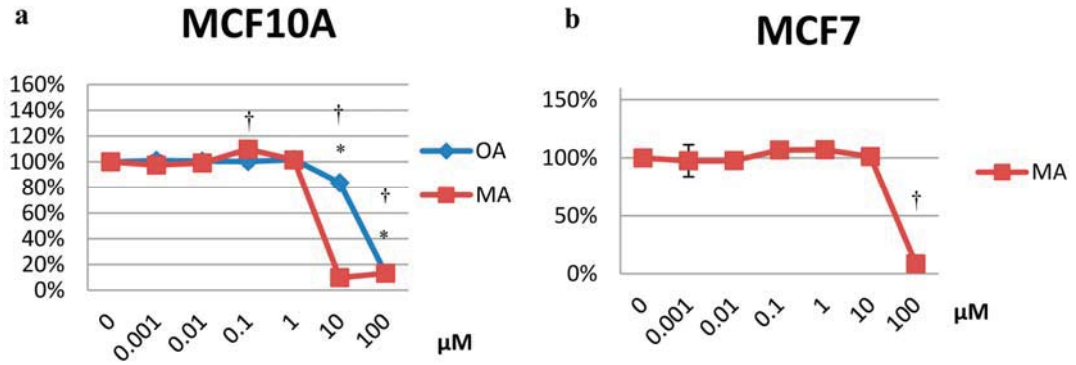
The triterpenoids are natural compounds that are widely distributed in the skin and seeds of different edible fruits, such as olives and grapes from *Vitis vinifera*. Oleanolic acid (OA) and maslinic acid (MA) are two of the main triterpenes found in these fruits; in addition, they are also present in both virgin olive oils and wine, especially red wine [1–7].

The traditional Mediterranean diet, characterized by the consumption of foods such as grapes, wine, must, raisins, olives and virgin olive oil, has been associated with a low incidence of breast cancer [8]. Current knowledge highlights the role of triterpenes in the prevention of certain cancers, including breast cancer [9–13]. Previously, it has been described that oleanolic acid and maslinic acid possess cardioprotective effects [14,15], anti-inflammatory effects [16,17], and antitumor properties in human prostate cancer cells [18], hepatocellular carcinoma cells [19], human pancreatic cells [20], and colon cancer cells, among others [21,22]. However, there are no studies about the potential chemopreventive effects of oleanolic and maslinic acids in human breast cells. We hypothesized that the chemopreventive effects of Mediterranean diet consumption against breast cancer may be due, at least in part, to the biological actions exerted by these compounds. To demonstrate this hypothesis, we have used the following well-characterized human breast cell lines: MCF10A human mammary epithelial cells, highly invasive MDA-MB-231 human breast cancer cells, and finally, minimally invasive MCF7 human breast cancer cells.

2. Results

2.1. Cytotoxicity Effects

The results are expressed as the percentage of cell survival with respect to the untreated control, which was set as 100%. For MCF10A cells, both OA and MA at 10 and 100 μM promoted cell death (cell survival was 83% and 13% for OA and 9% and 13% for MA, respectively) (Figure 1a). For MCF7 cells, MA induced a strong cytotoxic effect at 100 μM (8% survival) (Figure 1b). MDA-MB-231 cells treated with the two acids showed a marked cytotoxic effect for OA or MA at 100 μM (68% and 17% survival, respectively). MA concentrations between 0.01 μM and 10 μM appeared to promote cell survival (Figure 1c).



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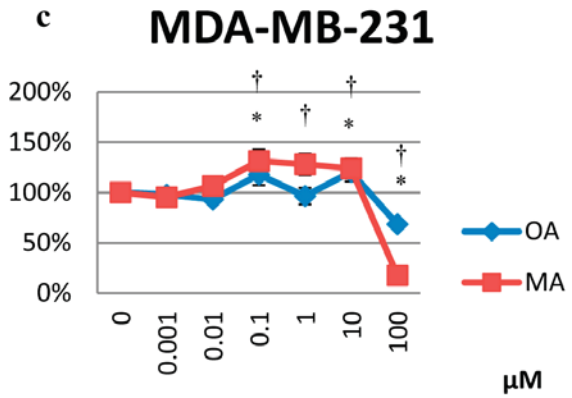
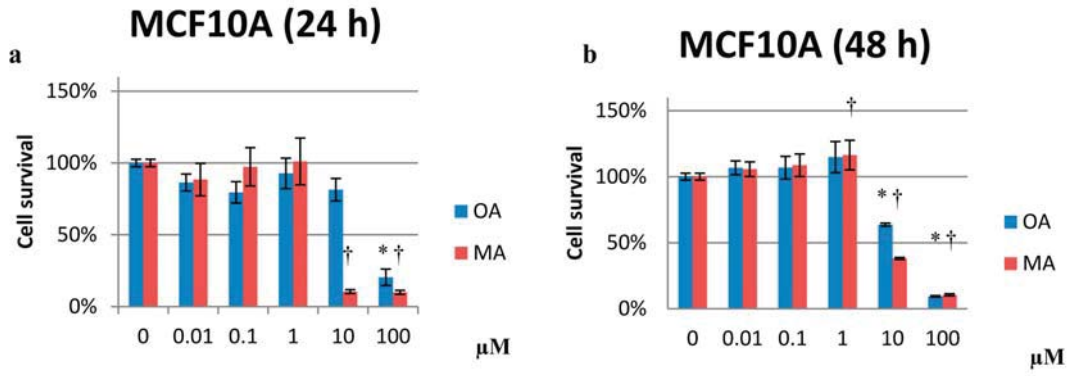


Figure 1. Cytotoxicity of OA and MA from 0.001 µM to 100 µM in MCF10A cells (**a**), MCF7 cells (**b**) and MDA-MB-231 cells (**c**) at 24 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA and (†) for MA at $p < 0.05$ compared to the untreated control.

In the human mammary epithelial cells, both compounds were cytotoxic at the highest concentrations. However, for MCF7, which is a multi-drug-resistant cancer cell line, only MA was capable of promoting cell death. OA did not significantly promote cytotoxicity in MCF7 cells, according to our previous study [10]. Our results agree with Shan *et al.*, who showed that OA did not strongly inhibit the growth of MCF7 cells [23]. In MDA-MB-231 cells, other studies of different plant extracts (which contain OA) have described antiproliferative effects [24,25]. Ponou *et al.* showed that isolated OA did not promote cytotoxicity at a maximum concentration of 200 µM [26], while we observed cytotoxicity at 100 µM.

2.2. Effects on Proliferation

The results are expressed as the percentage of cell survival with respect to the untreated control, which was set as 100%. MA at 10 and 100 µM had antiproliferative effects for MCF10A cells at 24, 48, and 72 h (10%, 38% and 11% cell survival for 10 µM and 9%, 10% and 11% for 100 µM, respectively) (Figure 2).



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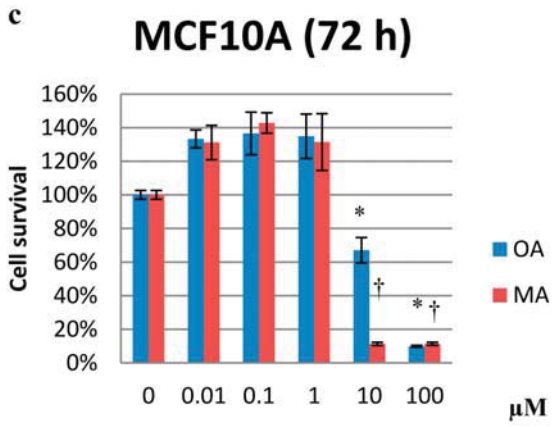
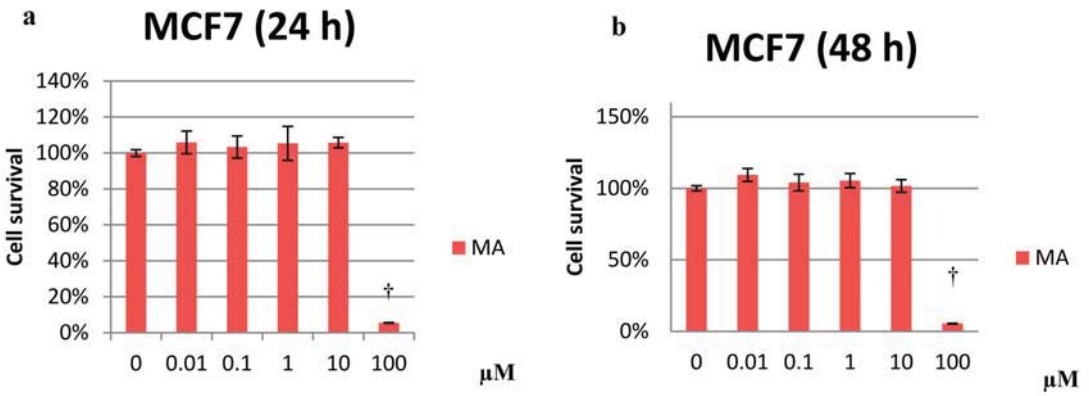


Figure 2. Percentage of cell proliferation in MCF10A cells after treatment with 0.01 µM to 100 µM OA or MA at 24 (**a**); 48 (**b**) and 72 h (**c**). Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA and (†) for MA at $p < 0.05$ with respect to the untreated control.

In MCF10A cells, OA inhibited proliferation at 10 and 100 µM after 48 and 72 h of treatment (~65% and 9% cell survival, respectively, at both time points) (Figure 2b,c). For MCF7 cells, MA was antiproliferative only at 100 µM (Figure 3). In MDA-MB-231 cells, OA inhibited proliferation in a dose-dependent manner at all treatment exposure times (Figure 4). Similarly, OA and MA at 10 and 100 µM inhibited proliferation in human mammary epithelial cells. However, at low concentrations, OA and MA appeared to increase the proliferation of the human mammary epithelial cells over time.

Notably, OA and MA were able to inhibit proliferation in a dose-dependent manner at all of the time exposures assayed in highly invasive breast cancer cells (MDA-MB-231) (Figure 4).



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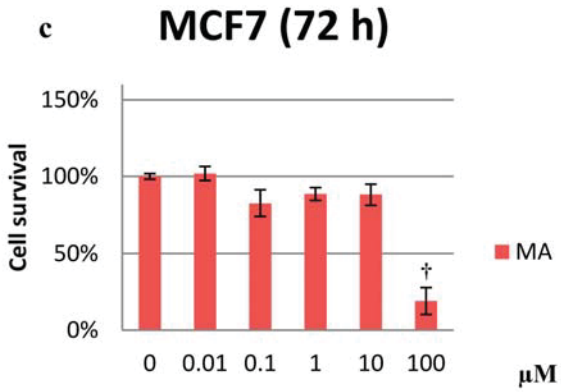


Figure 3. Percentage of cell proliferation in MCF7 cells after treatment with 0.01 µM to 100 µM MA at 24 (**a**); 48 (**b**) and 72 h (**c**). Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (†) for MA at $p < 0.05$ with respect to the untreated control.

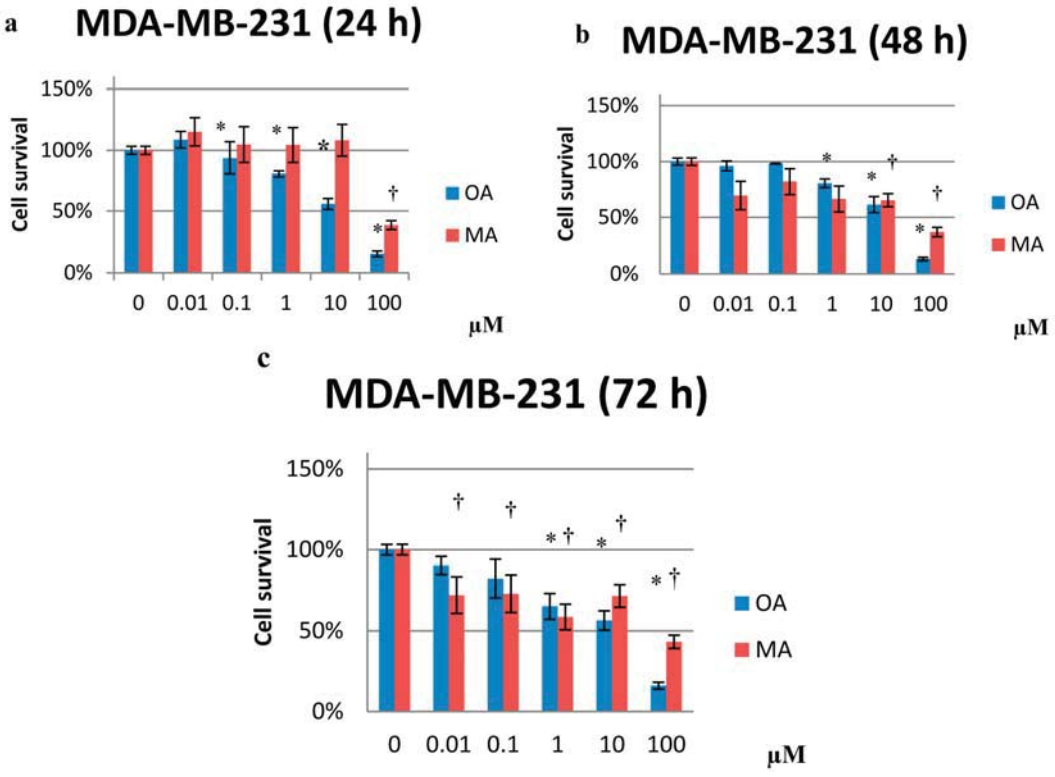


Figure 4. Percentage of cell proliferation in MDA-MB-231 cells after treatment with 0.01 µM to 100 µM OA or MA for 24 (**a**); 48 (**b**) and 72 h (**c**). Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA and (†) for MA at $p < 0.05$ with respect to the untreated control.

2.3. Effects on the Cell Cycle

The results are expressed as the percentage of cells in the different phases of the cell cycle. For MCF10A cells, OA treatment resulted in an increase in cells in the G0/G1 phase at 10 μM with respect to the control and a decrease in the G2/M phase. MA treatment resulted in a dramatic increase in the sub-G0/G1 phase at 10 μM (65%) with respect to the control (0.4%), and consequently resulted in a decrease in the other phases. At 10 μM, both compounds affected the cell cycle of MCF10A cells (Table 1).

Table 1. Distribution of cells in phases of the cell cycle for MDA-MB-231 and MCF10A cells treated with OA and MA at 0.1 μM, 1 μM, and 10 μM at 24 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) at $p < 0.05$ with respect to the untreated control.

	Percentage of Cells							
	MDA-MB-231				MCF10A			
	Sub-G0/G1	**G0/G1**	**S**	**G2/M**	**Sub-G0/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 *

We have discussed the importance of the different concentrations of treatments used in experiments [27], and our results show that high concentrations of these compounds could promote cell death in human mammary epithelial cells. For MDA-MB-231 cells, OA treatment resulted in a decrease in the number of cells in G0/G1 and an increase in G2/M at 0.1 μM with respect to the control. At 10 μM, OA increased the number of cells in the G2/M phase with respect to the control. MA treatment did not result in a significant difference in MDA-MB-231 (Table 1) or MCF7 cells (data not shown). These results suggest that MA affects the cell cycle of MCF10A cells, increasing the Sub-G0/G1 ratio. This increase could be due to pro-apoptotic effects. To assess this apoptotic effect, our group studied the apoptosis-promoting effects of these compounds in the three breast cell lines.

2.4. Analysis of Apoptosis

The percentages of living, apoptotic, and necrotic cells are represented with respect to the total, which was set as 100% (Table 2).

For MCF10A cells, 10 μM OA resulted in a high percentage of apoptotic cells with respect to the control. MA at 10 μM increased the rate of apoptotic cells. For MDA-MB-231 cells, statistically significant differences were not found, but 1 μM OA resulted in a slight increase in the apoptotic cell rate (Table 2). MA treatment in MCF7 cells did not result in a significant difference with respect to the control (data not shown).

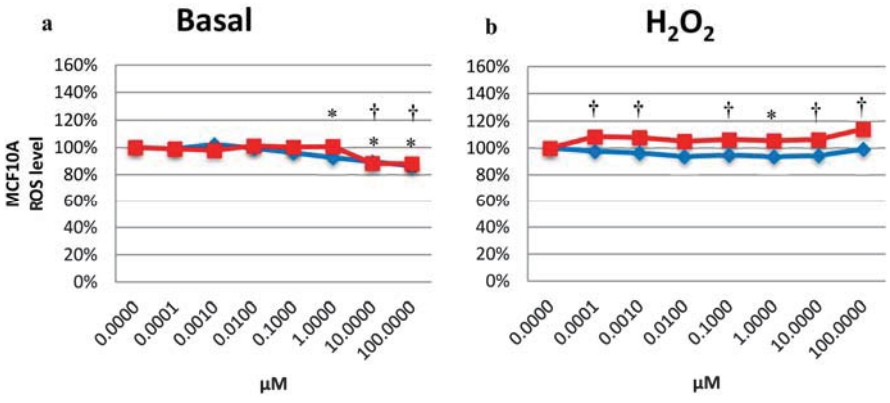
Table 2. Apoptosis of MDA-MB-231 and MCF10A cells treated with OA or MAS at 0.1 μM, 1 μM and 10 μM at 24 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) at $p < 0.05$ with respect to the untreated control.

	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

MA and OA at the highest concentrations caused apoptosis in MCF10A cells, while concentrations lower than 10 μM did not appear to promote apoptosis. However, in both breast cancer cell lines, neither OA nor MA produced a dramatic increase in apoptosis; only 1 μM OA slightly increased the apoptotic ratio in MDA-MB-231 cells. This slight increase could correspond with the proliferation observed, where OA decreased the proliferation in a dose-dependent manner over time.

2.5. Effects on the Intracellular ROS Level

In MCF10A cells treated with OA and MA, the levels of ROS decreased from 1 μM to 100 μM OA and from 10 to 100 μM for MA (Figure 5a). MA treatment in MCF7 cells increased the ROS levels in a dose-dependent manner (Figure 5c). In MDA-MB-231, OA treatment resulted in an increase in the ROS levels at 0.001 μM and 100 μM, while MA did not alter the ROS levels at any concentration tested (Figure 5d).



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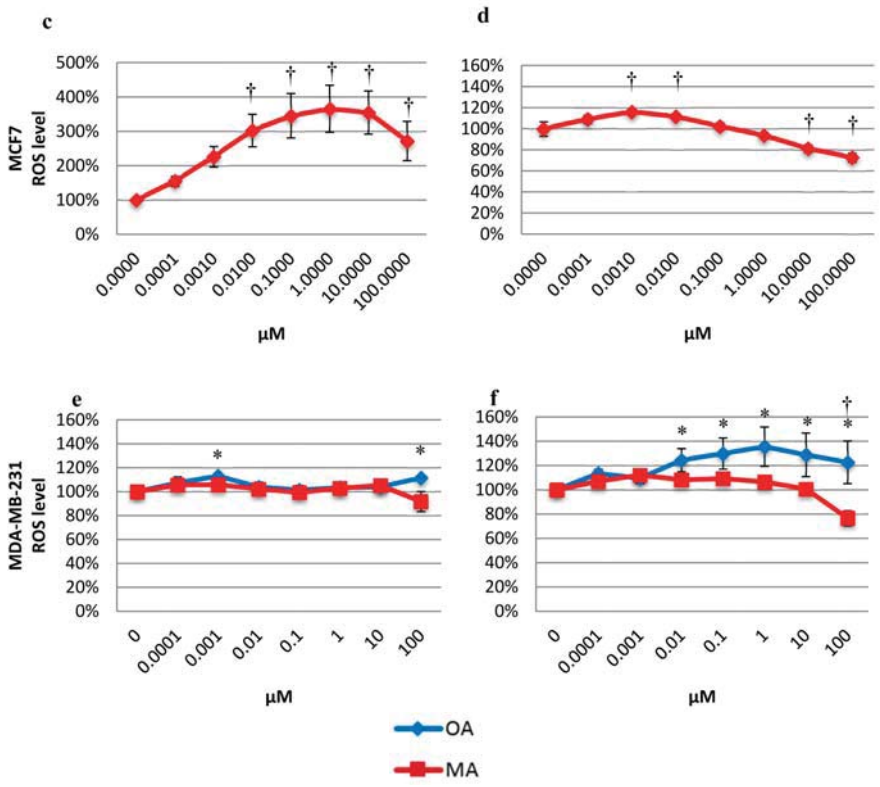

Figure 5. The ROS levels present in MCF10A cells in the basal state (**a**) and with H_2O_2 burst (**b**); in MCF7 cells in the basal state (**c**) and with H_2O_2 burst (**d**); and in MDA-MB-231 cells in the basal state (**e**) and with H_2O_2 burst (**f**) after treatment with OA or MA from 0.0001 µM to 100 µM for 4 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA and (†) for MA at $p < 0.05$ with respect to the untreated control.

To induce intracellular oxidative stress, H_2O_2 was added before the fluorescence measurement. Figure 5b shows a decrease in the ROS levels in MCF10A cells for OA; however, this difference was statistically significant only at 1 µM. MA treatment increased the ROS levels in MCF10A cells at almost all concentrations (Figure 5b). For MCF7 cells, MA appeared to increase the ROS levels at lower concentrations (Figure 5d). The ROS levels in MDA-MB-231 cells increased with OA treatment from 0.01 µM to 100 µM, while MA treatment did not result in any statistically significant differences with respect to the control, except for 100 µM, which decreased the ROS level (Figure 5f).

OA had a protective effect on MCF10A cells. It diminished ROS levels in the basal state, and when oxidative stress was induced, OA continued protecting the cells, reducing their sensitivity 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 (in up to 50%) [28]. In this way, OA could act like an antioxidant, protecting cells in an oxidative stress microenvironment, which could promote carcinogenesis [27,28]. To assess this theory, our group studied the effects of OA and MAS in H_2O_2-induced DNA damage.

Although MA did not have this effect in MCF10A cells, it resulted in a strong increase in oxidative stress in MCF7 cells in a dose-dependent manner, which continued when oxidative stress was induced. In MDA-MB-231 cells, both compounds exerted this pro-oxidative effect. In the basal state, lower concentrations of OA appeared to increase the oxidative stress in MDA-MB-231 cells. In addition, when intracellular oxidative stress was induced by adding H_2O_2, OA dramatically increased the oxidative stress, approximately 30% more than the control. MA had the same effect but to a lesser extent. Therefore, OA had a protective role against oxidative stress in human mammary epithelial cells, while it had a pro-oxidant role in the highly invasive breast cancer cells. This pro-oxidant role in breast cancer cells could be important, considering that high enough levels of ROS may inhibit carcinogenesis by enhancing p53 expression and inducing apoptosis in tumour cells [28]. To corroborate these effects in ROS levels, antioxidant catalase (CAT) enzyme activity was evaluated.

2.6. Determination of CAT Activity

The activity of CAT measured in MCF10A cells after OA and MA treatment showed no statistically significant differences with respect to the control (Figure 6a).

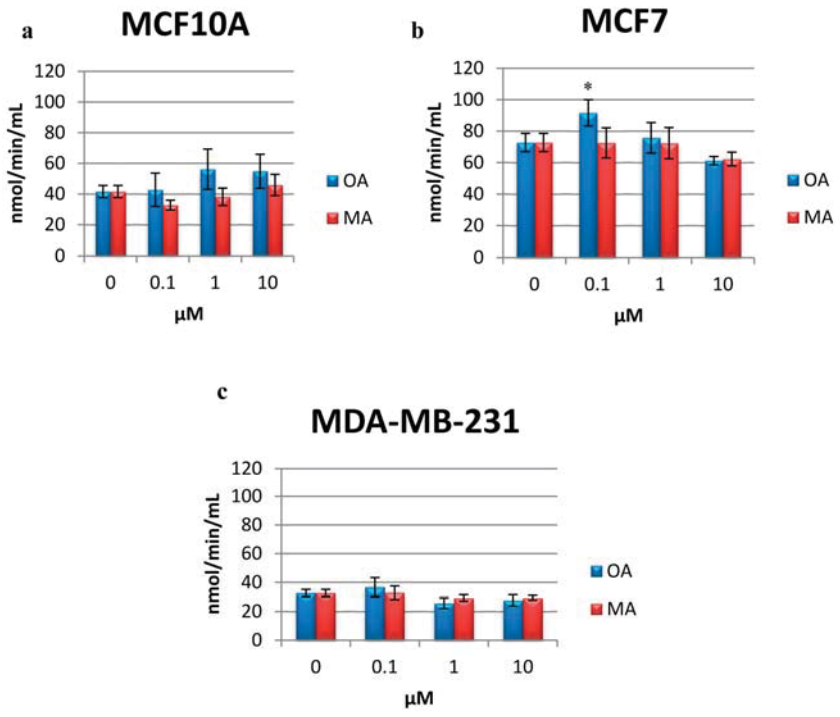

Figure 6. CAT activity in MCF10A cells (**a**); MCF 7 cells (**b**) and MDA-MB-231 cells (**c**) treated with OA or MA at 0.1 µM, 1 µM and 10 µM for 4 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA at $p < 0.05$ with respect to the untreated control.

In MCF7 cells, 0.1 µM OA increased CAT production significantly but appeared to decrease its production at higher concentrations. While 0.1 µM of OA was not assayed by Allouche, *et al.* [10], 1 µM

and 10 μM OA decreased the ROS levels; this could be related with the levels of CAT found in MCF7 cells in the present study (Figure 6b). MA did not alter the activity of CAT with respect to the control in MCF7 cells (Figure 6b).

Although there were no statistically significant differences in treated MDA-MB-231 cells, there was a slight decrease in the activity of CAT at 1 and 10 μM OA (Figure 6c).

2.7. Effects on H_2O_2-Induced DNA Damage

To study the protective effect of these triterpenes against induced DNA injury, H_2O_2 was used to promote single-strand DNA breaks. The results are expressed as 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 the capacity for self-repair [29].

Our results showed that for MCF10A cells, 1 μM OA protected against H_2O_2 injury to DNA, producing less DNA breaks than the control (Figure 7a). MA had the same effect at 10 μM, but it must be noted that at this concentration, MA was pro-apoptotic for the human mammary epithelial cells. Therefore, this result was likely due to cells that remained alive in the cytotoxicity and proliferation assay and were not affected by MA.

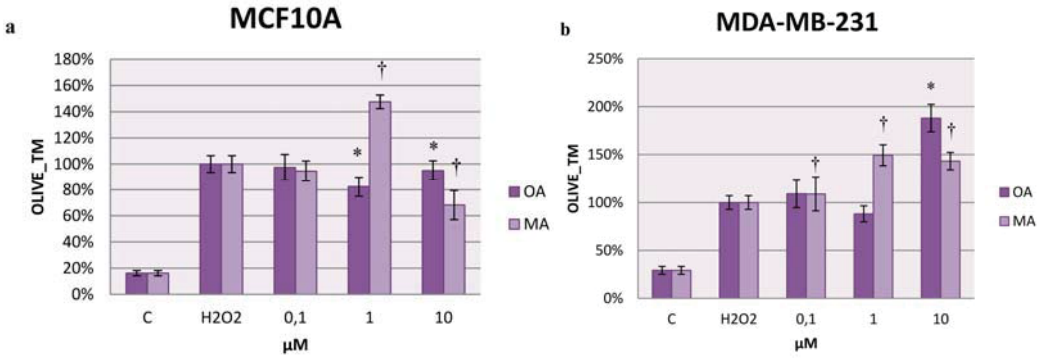

Figure 7. Olive_TM represented in MCF10A cells (**a**) and MDA-MB-231 cells (**b**) treated with OA or MA at 0.1 μM, 1 μM, and 10 μM for 4 h. Values represent the mean ± SEM of three independent experiments. Statistically significant differences are represented by (*) for OA and (†) for MA at $p < 0.05$ with respect to the H_2O_2 control.

Although 1 μM MA did not have pro-apoptotic effects, it appeared to promote damage to DNA, which supports the results obtained for the detection of ROS levels after H_2O_2 addition. MA could act like a pro-oxidant in these cells, increasing the ROS levels in the first moments of treatment and resulting in damage to the DNA after addition of H_2O_2, consistent with our results. However, the effect of MA at this concentration did not remain the same over time, as the proliferation results have shown.

In the MCF7 cells, MA did not show significant differences with respect to the control (data not shown). In the MDA-MB-231 cells, OA promoted an increase in Olive_TM at 10 μM, and although it was not statistically significant, this increase also occurred at 0.1 μM. MA induced more injury to the DNA,

increasing the Olive_TM at all concentrations tested in MDA-MB-231 cells (Figure 7b). Consequently, for highly invasive breast cancer cells, with only 4 h of treatment, both compounds promoted a high extent of damage to the DNA. Therefore, the cytotoxic effects of oleanolic acid observed in MDA-MB-231 cells appeared to be connected with the increase observed in the ROS levels that in turn promoted damage to the DNA.

3. Discussion

OA and MA are two triterpenes present in several plants, including grapevines and olive trees and consequently in their fruits. It is well known that the Mediterranean diet plays a role in preventing breast cancer [8], and these foods are typically found in this diet. Several studies have suggested the antitumoral properties of AO and MA, but until now, there has not been scientific data about their chemopreventive activity in human breast cancer and in human mammary epithelial cells. The present study is focused on the effects of these two natural compounds on human breast cancer cells and on human mammary epithelial cells, which never were studied before.

The results obtained show that MA inhibited the growth of minimally invasive MCF7 human breast cancer cells only at the highest concentration tested. Thus, MA treatment does not alter the cell cycle or induce apoptosis at the concentrations used previously by our group [10] or in the present study. However, Janicke, *et al.* [30] indicated that MCF7 cells have lost caspase-3 due to a 47-base-pair deletion within exon 3 of the CASP-3 gene, and this deletion is required for DNA fragmentation and phosphatidylserine expression on the cell surface. Accordingly, in the present study, MCF7 cells did not experience apoptosis, as indicated by flow cytometry, nor did they have changes in DNA fragmentation by the comet assay, but a decrease in cell proliferation was observed with OA treatment [10] and MA treatment. Thus, MA, which in turn promoted a dramatic increase in the ROS levels inside MCF7 cells, may promote their death but through a pathway distinct from apoptosis. In fact, an increase in ROS levels could contribute to cell death in cancer cells [31].

Indeed, MA can promote apoptosis in HT29 colon cancer cells through ROS generation [32,33]. Therefore, the connection between the ROS levels and cell death appears to be established. Our results demonstrate that OA and MA promote DNA damage in MDA-MB-231 cells. Further in-depth studies focusing on the molecular mechanism of the effects of OA and MA in breast cancer cells must be performed to confirm this. It must be noted that for several assays, the MCF7 cells were treated with high-purity MA (purity >98%) because the present study shows differences in MCF7 cells not reported in the previous study [10], where the purity of MA was lower (>80%).

OA has been recently described to be pro-apoptotic in oestrogen receptor-negative/progesterone receptor-negative/HER2-negative (ER−/PR−/Her2−) breast cancer cells [34], and patients with an ER− genotype are considered to have more aggressive, highly invasive breast cancer than patients with an ER+ genotype [35]. Chu, *et al.* described the action of BN107 (an extract with several terpenoidal saponins similar to OA), which promotes apoptosis in MCF10A (ER−) and in MDA-MB-231 (ER−) cells [34]. They concluded that BN107 and OA are strong inhibitors of the Akt/mammalian target of rapamycin (mTOR) pathway, which could avoid chemoresistance development in ER− breast cancer cells. Our results show that although MCF10A cells are ER−, OA was not able to cause cell death at concentrations lower than 10 μM; at these concentrations, OA had antiproliferative effects in the highly invasive MDA-MB-231

human breast cancer cells. Based on these results, the effects of OA appear to not be related to ER expression; depending on the concentration used, OA is able to promote cell death in ER− cells (MDA-MB-231 and MCF10A) and ER+ cells (MCF7) [10].

OA has been shown to decrease the expression of Bcl-2 and increase the expression of Bax in B16F10 melanoma cells [36]. Perhaps OA exerts its effect in MDA-MB-231 cells by this pathway, which is related to oxidative mechanisms in the cell [27]. It is known that an increase in the ROS levels promotes apoptosis in breast cancer cells [37]. OA could increase the ROS levels in highly invasive cancer cells and could support the action of chemotherapies that increase oxidative stress inside cancer cells, which are usually used in more aggressive, highly invasive breast cancers.

Concentrations of OA and MA higher than 10 μM inhibited human mammary epithelial cell proliferation and promoted apoptosis over time, but lower concentrations even improved the proliferation of these human mammary epithelial cells. Hence, the concentration of the treatment used is an important consideration. Very few articles describe the bioavailability of these triterpenes in humans after intake [38–40]; but several studies confirm that OA can be absorbed (0.7% of total oral bioavailibity) by rats after intake, as well as MA which was observed even after 60 min of oral administration in rat's plasma [27]. However, the concentration within the cells after the metabolism of these compounds is not described yet. Nevertheless, the concentration at which they are present in virgin olive oil is less than in other types of olive oils [5].

Our results showed that OA acts like an antioxidant in human mammary epithelial cells (MCF10A) *in vitro*. OA may decrease the oxidative stress of cells by enzymatic CAT activation. Furthermore, when oxidative stress was induced, the cells treated with OA had decreased levels of oxidative stress compared to the untreated cells. The irreversible injuries to DNA and proteins caused by oxidative stress are usually prevented by antioxidants [28]; along these lines, OA acts as an antioxidant for MCF10A cells, protecting the cells against oxidative DNA damage. Moreover, OA inhibited proliferation in MDA-MB-231 cells (highly invasive human breast cancer cells).

For these reasons, we might consider that OA could have potential chemopreventive activity in human breast cancer: at low concentrations, OA is a natural compound that acts as an antioxidant and prevents oxidative DNA damage in human mammary epithelial cells. Additionally, it has antiproliferative effects in highly invasive cancer cells. This compound could be used as an adjuvant in breast cancer oxidative therapies, where it could maximize the effects of chemotherapy while protecting human mammary epithelial cells against the oxidative effects of cancer therapy. However, pharmacologic effects of OA have to be studied before assure this.

Nevertheless, extreme caution should be applied in the extrapolation of the present *in vitro* results to potential clinical effects in humans. Further studies are needed to confirm both the chemopreventive capacity of OA and the differential mechanism of action on human mammary epithelial *vs* breast cancer cells suggested by the present study.

4. Experimental Section

4.1. 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; 100× non-essential amino acid mixture (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). Dry methanol (max 0.005%) and absolute ethanol PRS were purchased from Panreac Quimica S.L.U. (Barcelona, Spain). The 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 Pharmingen (San Diego, CA, USA). The Annexin-V FITC kit was purchased from 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).

4.2. 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 MCF10A (ATCC® Number: CRL-10317™) human mammary 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. Cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells were routinely subcultured using TrypLE Express solution. Cells in the exponential growth phase were used for all experiments. Except for the assays which specify differently, the cells were treated with 0.1 μM, 1 μM, or 10 μM oleanolic acid (OA) and maslinic acid (MA) for 4 h.

4.3. Cytotoxicity Assay

Cell survival, measured as the cellular growth of the treated cells *vs.* the untreated controls, was carried out in MCF10A, MCF7 and MDA-MB-231 cells using an XTT-based assay according to Scudiero, *et al.* [41], with some modifications. Briefly, cells were seeded into 96-well culture plates in a total volume of 100 μL per well (5×10^3 cells/well for MDA-MB-231 and MCF7 cells and 2.5×10^3 cells/well for MCF10A cells). After an overnight incubation to allow for cell attachment, 100 μL of fresh medium was added containing increasing concentrations from 0.001 μM to 100 μM OA or MA. After 24 h, the cells were incubated with XTT in Phenol-Red-free RPMI medium for 3 h at 37 °C with 5% CO_2, and the absorbance was measured at a 450 nm wavelength (620 nm as a reference) in a plate reader (TECAN GENios Plus). The cell viability was calculated using the formula:

$$\% \text{ viable cells} = [A(\text{treated cells})/A(\text{control})] \times 100 \quad (1)$$

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, the cells were treated with EtOH at the highest concentration of OA and MA used.

4.4. Cell Proliferation Assay

Cell proliferation, measured as the cellular growth of the treated cells *vs.* the untreated controls, was carried out using a CellTiter-Blue Cell Viability Assay. Briefly, the cells were seeded into 96-well culture plates at 2×10^3 cells/well for MCF7 cells, 1×10^3 cells/well for MDA-MB-231 cells and 0.5×10^3 cells/well for MCF10A cells. After an overnight incubation to allow for cell attachment, the medium was removed and replaced with fresh medium containing OA or MA from 0.01 μM to 100 μM. The 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, the 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). The cell viability was calculated using the formula:

$$\% \text{ viable cells} = [A(\text{treated cells})/A(\text{control})] \times 100 \quad (2)$$

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, the cells were treated with EtOH at the highest concentration of OA or MA used.

4.5. Cell Cycle Assay

The cells were seeded in 12-well culture plates (1×10^5 cells/well for MDA-MB-231 and MCF7 cells and 0.5×10^5 cells/well for MCF10A cells) and incubated overnight to allow for cell attachment. Next, the cells were treated with 0.1 μM, 1 μM, or 10 μM OA or MA for 24 h; the cells were harvested with TrypLE Express and washed with 1× PBS (Ca^{2+}/Mg^{2+} free) (300× *g*, 10 min at 4 °C). Finally, the 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), the cells were analysed by flow cytometry (EPICS XL-MCL, Beckman Coulter, Spain). The FlowJo program (v5.7.2, FlowJo LLC data analysis software, Ashland, OR, USA) was used to calculate the percentage of cells in the G0/G1, S and G2/M phases. Each experiment was independently repeated at least three times.

4.6. Apoptosis Assay

The percentage of apoptotic cells was determined using a double staining assay with FITC-conjugated Annexin V and propidium iodide (PI). Briefly; the cells were seeded in 12-well culture plates (1×10^5 cells/well for MDA-MB-231 and MCF7 cells and 0.5×10^5 cells/well for MCF10A cells) and incubated overnight to allow for cell attachment. After cell exposure to OA or MA at 0.1 μM, 1 μM, or 10 μM for 24 h; the cells were harvested with TrypLE Express; washed twice in cold 1× PBS (Ca^{2+}/Mg^{2+} free) (300× *g*; 10 min at 4 °C) and resuspended in 100 μL of Annexin Binding Buffer. The 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; the cells were treated with 1 μM camptothecin (CPT). Each experiment was independently repeated at least three times.

4.7. Detection of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) levels were measured after OA or MA treatment using the cell-permeable fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA), as previously described by Warleta, *et al.* [11], with some modifications. Briefly, the cells were seeded on 96-well plates (5×10^3 cells/well for MDA-MB-231 and MCF7 cells and 2.5×10^3 cells/well for MCF10A cells), and after incubation with the treatments, DCFH-DA (100 μM) was added for 30 min at 37 °C with 5% CO_2. The fluorescence was read in a plate reader for 30 min (Ex. λ485/Em. λ535, Gain 60). The intracellular ROS level percentage was calculated as follows:

$$F = [(F(t = 30) – F(t = 0))/F(t = 0) \times 100] \quad (3)$$

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 damages DNA [42]. To evaluate the protective capacity of OA and MA against induced oxidative stress, 500 μM H_2O_2 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 media (MEM and HuMEC).

4.8. Determination of Catalase (CAT) Activity

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

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

The cells were seeded into 12-well plates (1×10^5 cells/well for MDA-MB-231 cells and MCF7 cells and 0.5×10^5 cells/well for MCF10A cells) and incubated overnight for cell attachment. Then, the cells were treated with OA and MA. Finally, the cells were scraped and washed twice (300× *g*, 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free) and suspended in 1 mL of cold 1× PBS. To evaluate the ability of OA and MA to protect against oxidative DNA damage, the cells were exposed for 10 min to 50 μM H_2O_2 at 4 °C. After that, the comet assay was performed according to Warleta, *et al.* [11].

4.10. 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 wavelengths using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK). Twenty-five cell images were randomly characterized per sample using 20× magnification. The relative fluorescence between the head and tail through the olive tail moment (Olive_TM) was used to determine

DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail:

$$\text{Olive\_TM} = [(\text{tail (mean)} - \text{head (mean)}) \times \text{tail (\% DNA)}]/100 \quad (4)$$

4.11. Statistical Analysis

The results are displayed as the mean of at least three independent experiments (± SEM), and the results are expressed as a percentage relative to the untreated control, which was set as 100%. Statistical analysis was performed using a 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, VA, USA) was used for the statistical analysis.

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Author Contributions

C.S.-Q. and A.L.-B. carried out sample processing. C.S.-Q. and J.J.G. wrote the manuscript. J.J.G. conceived the study. All authors read and approved the final manuscript.

Conflicts of Interest

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

References

1. Zhang, Y.; Jayaprakasam, B.; Seeram, N.P.; Olson, L.K.; DeWitt, D.; Nair, M.G. Insulin Secretion and Cyclooxygenase Enzyme Inhibition by Cabernet Sauvignon Grape Skin Compounds. *J. Agric. Food Chem.* **2004**, *52*, 228–233.
2. Glensk, M.; Glinski, J.A.; Wlodarczyk, M.; Stefanowicz, P. Determination of Ursolic and Oleanolic Acid in Sambuci Fructus. *Chem. Biodivers.* **2014**, *11*, 1939–1944.
3. Szakiel, A.; Mroczek, A. Distribution of Triterpene Acids and their Derivatives in Organs of Cowberry (*Vaccinium vitis-idaea* L.) Plant. *Acta Biochim. Pol.* **2007**, *54*, 733–740.
4. Szakiel, A.; Paczkowski, C.; Koivuniemi, H.; Huttunen, S. Comparison of the Triterpenoid Content of Berries and Leaves of Lingonberry *Vaccinium vitis-idaea* from Finland and Poland. *J. Agric. Food Chem.* **2012**, *60*, 4994–5002.
5. Allouche, Y.; Jimenez, A.; Uceda, M.; Aguilera, M.P.; Gaforio, J.J.; Beltran, G. Triterpenic Content and Chemometric Analysis of Virgin Olive Oils from Forty Olive Cultivars. *J. Agric. Food Chem.* **2009**, *57*, 3604–3610.

6. Pensec, F.; Paczkowski, C.; Grabarczyk, M.; Wozniak, A.; Benard-Gellon, M.; Bertsch, C.; Chong, J.; Szakiel, A. Changes in the Triterpenoid Content of Cuticular Waxes during Fruit Ripening of Eight Grape (*Vitis vinifera*) Cultivars Grown in the Upper Rhine Valley. *J. Agric. Food Chem.* **2014**, *62*, 7998–8007.
7. Yunoki, K.; Sasaki, G.; Tokuji, Y.; Kinoshita, M.; Naito, A.; Aida, K.; Ohnishi, M. Effect of Dietary Wine Pomace Extract and Oleanolic Acid on Plasma Lipids in Rats Fed High-Fat Diet and its DNA Microarray Analysis. *J. Agric. Food Chem*. **2008**, *56*, 12052–12058.
8. Buckland, G.; Travier, N.; Cottet, V.; Gonzalez, C.A.; Lujan-Barroso, L.; Agudo, A.; Trichopoulou, A.; Lagiou, P.; Trichopoulos, D.; Peeters, P.H.; *et al.* Adherence to the Mediterranean Diet and Risk of Breast Cancer in the European Prospective Investigation into Cancer and Nutrition Cohort Study. *Int. J. Cancer* **2013**, *132*, 2918–2927.
9. Patlolla, J.M.; Rao, C.V. Triterpenoids for Cancer Prevention and Treatment: Current Status and Future Prospects. *Curr. Pharm. Biotechnol*. **2012**, *13*, 147–155.
10. Allouche, Y.; Warleta, F.; Campos, M.; Sanchez-Quesada, C.; Uceda, M.; Beltran, G.; Gaforio, J.J. 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.* **2011**, *59*, 121–130.
11. Warleta, F.; Campos, M.; Allouche, Y.; Sanchez-Quesada, C.; Ruiz-Mora, J.; Beltran, G.; Gaforio, J.J. Squalene Protects against Oxidative DNA Damage in MCF10A Human Mammary Epithelial Cells but Not in MCF7 and MDA-MB-231 Human Breast Cancer Cells. *Food Chem. Toxicol*. **2010**, *48*, 1092–1100.
12. Warleta, F.; Quesada, C.S.; Campos, M.; Allouche, Y.; Beltran, G.; Gaforio, J.J. Hydroxytyrosol Protects Against Oxidative DNA Damage in Human Breast Cells. *Nutrients* **2011**, *3*, 839–857.
13. Sanchez-Quesada, C.; Lopez-Biedma, A.; Gaforio, J.J. The Differential Localization of a Methyl Group Confers a Different Anti-Breast Cancer Activity to Two Triterpenes Present in Olives. *Food Funct.* **2015**, *6*, 248–255.
14. Allouche, Y.; Beltran, G.; Gaforio, J.J.; Uceda, M.; Mesa, M.D. Antioxidant and Antiatherogenic Activities of Pentacyclic Triterpenic Diols and Acids. *Food Chem. Toxicol*. **2010**, *48*, 2885–2890.
15. Senthil, S.; Sridevi, M.; Pugalendi, K.V. Cardioprotective Effect of Oleanolic Acid on Isoproterenol-Induced Myocardial Ischemia in Rats. *Toxicol. Pathol*. **2007**, *35*, 418–423.
16. Graham, V.S.; Lawson, C.; Wheeler-Jones, C.P.; Perona, J.S.; Ruiz-Gutierrez, V.; Botham, K.M. Triacylglycerol-Rich Lipoproteins Derived from Healthy Donors Fed Different Olive Oils Modulate Cytokine Secretion and Cyclooxygenase-2 Expression in Macrophages: The Potential Role of Oleanolic Acid. *Eur. J. Nutr*. **2012**, *51*, 301–309.
17. Li, C.; Yang, Z.; Zhai, C.; Qiu, W.; Li, D.; Yi, Z.; Wang, L.; Tang, J.; Qian, M.; Luo, J.; *et al.* Maslinic Acid Potentiates the Anti-Tumor Activity of Tumor Necrosis Factor Alpha by Inhibiting NF-kappaB Signaling Pathway. *Mol. Cancer*. **2010**, *9*, doi:10.1186/1476-4598-9-73.
18. Park, S.Y.; Nho, C.W.; Kwon, D.Y.; Kang, Y.H.; Lee, K.W.; Park, J.H. Maslinic Acid Inhibits the Metastatic Capacity of DU145 Human Prostate Cancer Cells: Possible Mediation via Hypoxia-Inducible Factor-1alpha Signalling. *Br. J. Nutr*. **2013**, *109*, 210–222.

19. Wang, X.; Bai, H.; Zhang, X.; Liu, J.; Cao, P.; Liao, N.; Zhang, W.; Wang, Z.; Hai, C. Inhibitory Effect of Oleanolic Acid on Hepatocellular Carcinoma via ERK-p53-Mediated Cell Cycle Arrest and Mitochondrial-Dependent Apoptosis. *Carcinogenesis* **2013**, *34*, 1323–1330.
20. Wei, J.; Liu, M.; Liu, H.; Wang, H.; Wang, F.; Zhang, Y.; Han, L.; Lin, X. Oleanolic Acid Arrests Cell Cycle and Induces Apoptosis via ROS-Mediated Mitochondrial Depolarization and Lysosomal Membrane Permeabilization in Human Pancreatic Cancer Cells. *J. Appl. Toxicol.* **2013**, *33*, 756–765.
21. Rufino-Palomares, E.E.; Reyes-Zurita, F.J.; Garcia-Salguero, L.; Mokhtari, K.; Medina, P.P.; Lupianez, J.A.; Peragon, J. Maslinic Acid, a Triterpenic Anti-Tumoural Agent, Interferes with Cytoskeleton Protein Expression in HT29 Human Colon-Cancer Cells. *J. Proteomics* **2013**, *83*, 15–25.
22. Sanchez-Tena, S.; Reyes-Zurita, F.J.; Diaz-Moralli, S.; Vinardell, M.P.; Reed, M.; Garcia-Garcia, F.; Dopazo, J.; Lupianez, J.A.; Gunther, U.; Cascante, M. Maslinic Acid-Enriched Diet Decreases Intestinal Tumorigenesis in Apc(Min/+) Mice through Transcriptomic and Metabolomic Reprogramming. *PLoS ONE* **2013**, *8*, e59392.
23. Shan, J.Z.; Xuan, Y.Y.; Ruan, S.Q.; Sun, M. Proliferation-Inhibiting and Apoptosis-Inducing Effects of Ursolic Acid and Oleanolic Acid on Multi-Drug Resistance Cancer Cells *in Vitro*. *Chin. J. Integr. Med.* **2011**, *17*, 607–611.
24. He, X.; Wang, Y.; Hu, H.; Zhang, Z. *In Vitro* and *in Vivo* Antimammary Tumor Activities and Mechanisms of the Apple Total Triterpenoids. *J. Agric. Food Chem.* **2012**, *60*, 9430–9436.
25. Chakravarti, B.; Maurya, R.; Siddiqui, J.A.; Bid, H.K.; Rajendran, S.M.; Yadav, P.P.; Konwar, R. *In Vitro* Anti-Breast Cancer Activity of Ethanolic Extract of Wrightia Tomentosa: Role of Pro-Apoptotic Effects of Oleanolic Acid and Urosolic Acid. *J. Ethnopharmacol.* **2012**, *142*, 72–79.
26. Ponou, B.K.; Teponno, R.B.; Ricciutelli, M.; Nguelefack, T.B.; Quassinti, L.; Bramucci, M.; Lupidi, G.; Barboni, L.; Tapondjou, L.A. Novel 3-Oxo- and 3,24-Dinor-2,4-Secooleanane-Type Triterpenes from Terminalia Ivorensis A. *Chem. Biodivers.* **2011**, *8*, 1301–1309.
27. Sanchez-Quesada, C.; Lopez-Biedma, A.; Warleta, F.; Campos, M.; Beltran, G.; Gaforio, J.J. Bioactive Properties of the Main Triterpenes found in Olives, Virgin Olive Oil, and Leaves of *Olea europaea*. *J. Agric. Food Chem.* **2013**, *61*, 12173–12182.
28. Saeidnia, S.; Abdollahi, M. Antioxidants: Friends or Foe in Prevention or Treatment of Cancer: The Debate of the Century. *Toxicol. Appl. Pharmacol.* **2013**, *271*, 49–63.
29. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells. *Exp. Cell Res.* **1988**, *175*, 184–191.
30. Janicke, R.U.; Sprengart, M.L.; Wati, M.R.; Porter, A.G. Caspase-3 is required for DNA Fragmentation and Morphological Changes Associated with Apoptosis. *J. Biol. Chem.* **1998**, *273*, 9357–9360.
31. Bonnet, S.; Archer, S.L.; Allalunis-Turner, J.; Haromy, A.; Beaulieu, C.; Thompson, R.; Lee, C.T.; Lopaschuk, G.D.; Puttagunta, L.; Bonnet, S.; *et al.* A Mitochondria-K^+ Channel Axis is Suppressed in Cancer and its Normalization Promotes Apoptosis and Inhibits Cancer Growth. *Cancer Cell.* **2007**, *11*, 37–51.
32. Reyes-Zurita, F.J.; Rufino-Palomares, E.E.; Lupianez, J.A.; Cascante, M. Maslinic Acid, a Natural Triterpene from *Olea europaea* L., Induces Apoptosis in HT29 Human Colon-Cancer Cells via the Mitochondrial Apoptotic Pathway. *Cancer Lett.* **2009**, *273*, 44–54.

33. Reyes-Zurita, F.J.; Pachon-Pena, G.; Lizarraga, D.; Rufino-Palomares, E.E.; Cascante, M.; Lupianez, J.A. The Natural Triterpene Maslinic Acid Induces Apoptosis in HT29 Colon Cancer Cells by a JNK-p53-Dependent Mechanism. *BMC Cancer* **2011**, *11*, doi:10.1186/1471-2407-11-154.
34. Chu, R.; Zhao, X.; Griffin, C.; Staub, R.E.; Shoemaker, M.; Climent, J.; Leitman, D.; Cohen, I.; Shtivelman, E.; Fong, S. Selective Concomitant Inhibition of mTORC1 and mTORC2 Activity in Estrogen Receptor Negative Breast Cancer Cells by BN107 and Oleanolic Acid. *Int. J. Cancer* **2010**, *127*, 1209–1219.
35. Sheikh, M.S.; Garcia, M.; Pujol, P.; Fontana, J.A.; Rochefort, H. Why are Estrogen-Receptor-Negative Breast Cancers More Aggressive than the Estrogen-Receptor-Positive Breast Cancers? *Invasion Metastasis* **1994**, *14*, 329–336.
36. Pratheeshkumar, P.; Kuttan, G. Oleanolic Acid Induces Apoptosis by Modulating p53, Bax, Bcl-2 and Caspase-3 Gene Expression and Regulates the Activation of Transcription Factors and Cytokine Profile in B16F. *J. Environ. Pathol. Toxicol. Oncol*. **2011**, *30*, 21–31.
37. Vera-Ramirez, L.; Sanchez-Rovira, P.; Ramirez-Tortosa, M.C.; Ramirez-Tortosa, C.L.; Granados-Principal, S.; Lorente, J.A.; Quiles, J.L. Free Radicals in Breast Carcinogenesis, Breast Cancer Progression and Cancer Stem Cells. Biological Bases to Develop Oxidative-Based Therapies. *Crit. Rev. Oncol. Hematol*. **2011**, *80*, 347–368.
38. Song, M.; Hang, T.J.; Wang, Y.; Jiang, L.; Wu, X.L.; Zhang, Z.; Shen, J.; Zhang, Y. Determination of Oleanolic Acid in Human Plasma and Study of its Pharmacokinetics in Chinese Healthy Male Volunteers by HPLC Tandem Mass Spectrometry. *J. Pharm. Biomed. Anal*. **2006**, *40*, 190–196.
39. Rada, M.; Ruiz-Gutierrez, V.; Guinda, A. Determination of Triterpenic Acids in Human Serum by High-Performance Liquid Chromatography: Triterpenoid Interaction with Serum Protein. *J. Agric. Food Chem*. **2011**, *59*, 2308–2313.
40. Lozano-Mena, G.; Juan, M.E.; Garcia-Granados, A.; Planas, J.M. Determination of Maslinic Acid, a Pentacyclic Triterpene from Olives, in Rat Plasma by High-Performance Liquid Chromatography. *J. Agric. Food Chem*. **2012**, *60*, 10220–10225.
41. Scudiero, D.A.; Shoemaker, R.H.; Paull, K.D.; Monks, A.; Tierney, S.; Nofziger, T.H.; Currens, M.J.; Seniff, D.; Boyd, M.R. Evaluation of a Soluble tetrazolium/formazan Assay for Cell Growth and Drug Sensitivity in Culture using Human and Other Tumor Cell Lines. *Cancer Res*. **1988**, *48*, 4827–4833.
42. Lee, D.H.; Lim, B.S.; Lee, Y.K.; Yang, H.C. Effects of Hydrogen Peroxide (H_2O_2) on Alkaline Phosphatase Activity and Matrix Mineralization of Odontoblast and Osteoblast Cell Lines. *Cell Biol. Toxicol*. **2006**, *22*, 39–46.

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Selective Antitumor Activity of Pressurized Mango Leaf Extracts against Minimally and Highly Invasive Breast Cancer

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Abstract

Mango polyphenols have demonstrated antioxidant and antitumor activities on breast cancer cells, amongst others. However, data on the anticarcinogenic capacity of mango leaf have not been published to date. The effect of pressurized mango leaf extracts against breast cancer has been investigated along with the influence of the extraction solvent and phenolic content. Although mangiferin was the predominant compound in the extracts, the results show that the selective inhibitory activity against hormone-receptor positive (MCF7) and -negative (MDA-MB-231) breast cancer cells can be correlated with other minor polyphenols. Ethanolic and CO2-hydroalcoholic extracts contained high levels of gallotannins and they showed a cytotoxic effect on MCF7 cells. MDA-MB-231 cells were more susceptible to the hydroalcoholic extract and fractions, which have as common components methyl gallate and homomangiferin, and these presented the highest antioxidant effect. Extracts also showed protective effects against non-tumorigenic mammary epithelial cells, thus highlighting their potential use as chemopreventive agents.

1. Introduction

Breast cancer is one of the most common global threats to women at present. Therapies used to treat breast cancer are mainly based on the antitumoral effects of certain chemicals that promote the death of cancer and normal epithelial cells, with the consequent effects. Recent studies indicate that the use of natural compounds in cancer therapies could promote tumor death without damage to epithelial cells.[1-2] Indeed, authors have focused on the discovery of the antitumoral potential of several natural compounds that avoid secondary effects on non-tumorigenic cells.

Soy isoflavonegenistein,[3] squalene, lignans or triterpenes from olive oil,[4-7] and pomegranate[8] for example have been shown to exert antitumoral actions in breast cancer cells.

Mango polyphenols have also been highlighted as very promising chemoprevention agents. Mangiferin, present in high concentrations in leaves and bark, has exhibited anticarcinogenic activity against different types of cancers such as leukemic, colon, lung, cervical, brain and breast cancers.[9] It exerts anti-metastatic and anti-invasiveness activity in cancer cells by selective gene expression regulation of enzymes metalloproteinases (MMP) which play a key role in cell proliferation. It is also capable of inhibiting epithelial mesenchymal transition, a process linked to metastatic propensity that is characterized by the loss of cell adhesion.[10-12]

Other mango polyphenols such as quercetin, gallic acid and pentagalloylglucose have also exhibited interesting antitumor activity in highly invasive breast cancer cells by modulation of gene expression and reduction of MMP and kinase activity.[10,13-17] And mango extracts obtained from pulp, peel and bark have also shown anticancer activities against breast cancer cells and other type of cancers.[10,18-19] However, very few investigations have provided empirical data for the use of mango leaf extracts as a chemopreventive agent.

Novel techniques such as Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE) and Enhanced Solvent Extraction (ESE), which use green solvents, including water, ethanol and carbon dioxide, have been used to produce extracts and fractions from mango leaves with high content of polyphenols. These

techniques have the advantages of saving energy and solvent/time, thus avoiding evaporation steps that cause undesired degradation of active thermolabile compounds.[20-24]

The extracts from mango leaves obtained by PLE and ESE have shown higher antioxidant activity when compared to the well-known antioxidant α-tocopherol and these extracts contain high levels of phenolics such as mangiferin, gallic acid, quercetin, iriflophenones and gallotannins.[23] In addition, recent studies have demonstrated the anti-inflammatory, anti-diabetic and antioxidant properties of these compounds and their potential to prevent neurodegenerative diseases. These findings suggest the potential use of pressurized mango leaf extracts as chemopreventive agent.[23,26-28]

Therefore, bearing in mind the information outlined above, the antitumoral activity of pressurized mango leaf extracts was evaluated against human breast cancer cells for first time using different in vitro assays. The influence that the extraction process (PLE and ESE), solvent system and phenolic content of the extracts had on the antitumor activity of pressurized mango leaf extracts was evaluated. In addition, a fraction obtained by concurrent extraction with SC-CO_2 and hydroalcoholic mixtures was also analyzed. The levels of intracellular reactive oxygen species and cell cytotoxicity in MDA-MB-231 and MCF7 human breast cancer cells and MCF10A non-tumorigenic human breast epithelial cells treated with pressurized mango leaf extracts were determined and analyzed statistically.

2. Methods and materials

2.1. Materials

Mango (*Mangifera indica*, cv. Kent) leaves were provided by the Experimental Farm 'La Mayora', Superior Centre of Scientific Research (CSIC), Malaga, Spain. The leaves were collected in September 2013. All leaves were dried at room temperature to constant weight and the samples were frozen in the absence of light.

2.2. Chemicals and reagents

The following materials 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). Minimum essential medium with Eagle's salts (MEM) and fetal bovine serum (FBS) were obtained from PAA Laboratories GmbH (Pasching, Austria). HuMEC Ready Medium kit was obtained from Invitrogen (Eugene, OR). Culture plates and cell culture flasks were obtained from NUNC A/S (Roskilde, Denmark). The CellTiter Blue reagent was obtained from Promega Biotech Ibérica, SL (Madrid, Spain). Carbon dioxide (99.99%) was obtained from Abello-Linde S.A. (Barcelona, Spain). $K_2S_2O_8$ (CAS 7727-21-1) and the organic solvents HPLC grade ethanol, acetonitrile and formic acid were supplied by Panreac (Barcelona, Spain). The standard compounds gallic acid, methyl gallate (98%), 3,4-dihydroxybenzoic acid (≥97%), mangiferin (≥98%), quercetin 3-D-galactoside (≥97%), quercetin 3-β-D-glucoside (≥90%), quercetin 3-O-α-L-arabinopyranoside (≥95%), penta-O-galloyl-β-D-glucose hydrate (≥96%) and quercetin (≥98%) were supplied by Sigma-Aldrich (Steinheim, Germany). Ultrapure water (Milli-Q) was used.

2.3. Cell culture

Minimally invasive MCF7 human breast cancer cells (estrogen and progesterone receptor-positive), highly invasive MDA-MB-231 human breast cancer cells (estrogen and progesterone receptor-negative), and non-tumorigenic human breast epithelial cells (MCF10A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human mammary epithelial cells (MCF10A) were grown in HuMEC Ready Medium. Breast tumor cells (MCF7 and MDA-MB-231) were cultivated in Minimum Essential Medium (MEM) with Eagle's salts with L-glutamine supplemented with 1% non-essential amino acids (NEAA), 1% Hepes buffer, 1% sodium pyruvate and 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells in the exponential growth

phase with approximately 90–95% of confluence were used for all experiments.

2.4. Production of mango leaf extracts by high pressure techniques

Mango leaf extracts were obtained by high pressure extraction techniques: pressurized liquid extraction (PLE) and enhanced solvent extraction (ESE). The extraction tests were carried out in a high pressure system (Thar Technology, model SF100, Pittsburgh, PA, USA) provided with a thermostatted vessel (capacity 100 mL), two pumps with a maximum flow rate of 50 g/min (one for carbon dioxide and the other for liquid solvents), a back pressure regulator valve and a cyclonic separator, which allows periodic discharge of the extracted material. A schematic diagram of the equipment is shown in **Fig. 1**.

Different extraction solvents were analyzed as follows: pure ethanol (PET), water-ethanol 50:50 (PEW), CO_2-water-ethanol 50:25:25 (CEW) and a fraction obtained with water-ethanol 50:50 previously extracted with supercritical-CO_2 (SC-CO_2) (FEW). All extractions were carried out at a temperature of 100 °C, a pressure of 12 MPa, a flow rate of 10 g/min and with an extraction time of 3 h. Extraction conditions were fixed according to economic aspects and the results of a previous study in which high pressure extractions of mango leaves were optimized[23] (Fernández-Ponce et al.; 2015). After the extraction procedure was complete the solvent was evaporated and the extracts were dried and frozen prior to analysis.

2.5. Identification and quantification of phenolic compounds

The phenolic compounds present in pressurized mango leaf extracts were quantified by high performance liquid chromatography (HPLC). Analyses were performed using an Agilent HPLC series 1100 system (Agilent, Germany) equipped with a quaternary pump, an autosampler, and a UV/vis detector connected to HP ChemStation® software. Extracts were filtered prior to injection into the HPLC system with a volume of 20 μL. Phenolic compounds were separated on a Synergi Hydro-RP C18 column (150 mm × 3 mm i.d.; 4 μm) (Phenomenex,

USA) with a 4.0 mm × 2.0 mm i.d. C18 ODS guard column, and eluted at 0.6 mL/min with a mobile phase consisting of water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B). A linear gradient profile with the following proportions of solvent B [t (min), %B] was applied: (0, 0), (0.2, 0), (0.3, 7), (14.7, 8.5), (40, 19), (45, 33), (48, 50), (50, 95), (57, 0), (63, 0). Phenolic compounds were detected at 278 nm and identified according to the retention time and the elution behavior found in a previous characterization study of mango leaf extracts by high pressure liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS).[23] The quantification was carried out on the basis of calibration curves generated from the standard compounds: gallic acid, mangiferin, quercetin 3-β-D-glucoside, quercetin, 3-β-D-glucoside, quercetin 3-O-α-L-arabinopyranoside, quercetin aglycone and penta-O-galloyl glucose.

2.6. Evaluation of antitumor activity

The antitumor or chemopreventive activities of mango leaf extracts in breast cancer and mammary epithelial cells were evaluated according to the cellular cytotoxicity assay and the detection of intracellular reactive oxygen species using 2',7'-dichlorofluorescein diacetate (DCFH-DA). All extracts were dissolved in DMSO and then filtered. The following concentrations were used for cytotoxicity and DCFH-DA assays: 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL, 0.0001 mg/mL, 0.00001 mg/mL.

2.6.1. Cellular cytotoxicity assay

The cytotoxicity assay was performed using the CellTiter Blue® reagent according to the manufacturer's protocol with some modifications. Cells in the exponential phase were cultured in 96-well plates (5×10^3 cells/well for MDA-MB-231 and MCF7 or 2.5×10^3 cells/well for MCF10A). After overnight incubation to allow cell attachment, the medium was removed and replaced with fresh medium containing mango leaf extracts at the concentrations mentioned in section 2.6, and plates were incubated for a further 24 h. Finally, the CellTiter Blue was added. Fluorescence (Ex. λ_{485}/Em. λ_{595})

was measured with a TECAN GENios Plus microplate reader (Tecan Group Ltd.; Switzerland) after 3 h of incubation in darkness at 5% CO_2 and 37 °C. Viability was calculated by equation (1):

$$\%\, Viable\ cells = (A_{treated\ cells}/A_{control}) \times 100 \quad \text{(Eq. 1)}$$

Where A corresponds to the relative fluorescence units of each sample. All measurements were performed in quadruplicate in three independent replicates.

2.6.2. Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were detected using DCFH-DA as previously reported (Sánchez-Quesada et al.; 2015b). In brief, MCF10A (5.5×10^3 cells/well), MDA-MB-231 or MCF7 cells (7×10^3 cells/well) were cultured in 96 well plates for 24 h. After the addition of the concentrations of extracts tested the cells were incubated for an additional 24 h. DCFH-DA (100 μM) was then added and the plates were incubated for 30 min. After this time, fluorimetric measurements (Ex. λ_{485}/Em. λ_{535}) were made using a TECAN GENios Plus microplate reader.

The intracellular ROS percentage was calculated according to equation 2:

$$F = [(Ft_{30} - Ft_0)/Ft_0] \times 100 \quad \text{(Eq.2)}$$

Where Ft_0 is the fluorescence at t = 0 min and Ft30 is the fluorescence at t = 30 min.

It has been reported that the addition of H_2O_2 increases oxidative stress in cultured cells.[29] Therefore, in order to evaluate the protective capacity of mango leaf extracts against induced oxidative stress, H_2O_2 was added at 500 μM 30 minutes before fluorescence quantification. Both sets of experimental conditions were run in triplicate in three independent replicates.

2.7. Statistical analyses

Results are presented as mean (± SEM) as a percentage relative to control, which was considered as 100%. Statistical analysis was

performed using analysis of variance (ANOVA) followed by Fisher's LSD test. Values of $p < 0.05$ were considered significant. STATGRAPHICS Centurion XVI software (StatPoint Technologies, Inc.; Warrenton, VA, USA) was used for the statistical analysis.

3. Results and discussion

3.1. Chemical characterization of extracts obtained by high pressure extraction

Pressurized liquid extraction (PLE) and enhanced solvent extraction (ESE) were explored in this work to obtain mango leaf extracts with high contents of potential anticarcinogenic compounds. In addition, for the first time a fraction of mango leaf extracts obtained by concurrent extraction with SC-CO_2 and a subsequent pressurized hydroalcoholic mixture was studied.

The phenolic profile of the different pressurized mango leaf extracts are presented in **Table 1**. All contained phenolic acids (gallic acid), xanthones (mangiferin), galloylated benzophenones (iriflophenone 3-C-β-D-glucoside, iriflophenone 3-C-(2-O-p-hydroxybenzoyl)-β-D-glucoside and iriflophenone 3-C-(2-O-galloyl)-β-D-glucoside), flavonols (quercetin 3-D-galactoside, quercetin 3-β-D-glucoside, quercetin 3-O-xyloside and quercetin 3-O-α-L arabinopyranoside), and gallotannins (tetra- and penta-O-galloyl-glucose). Mangiferin was the predominant compound present in all extracts followed by iriflophenone-glucoside, whereas mangiferin was the second most abundant compound for the fraction obtained in subsequent extractions with pure CO_2 and the hydroalcoholic mixture.

Some differences were also observed for the other phenolic compounds. Homomangiferin and methyl gallate were identified in the hydroalcoholic fraction previously extracted with CO_2 (FEW) and in the hydroalcoholic extract (PEW), although the hydroalcoholic fraction (FEW) did not contain penta-O-galloyl-glucose. In contrast, the pressurized ethanolic extract (PET) and the ESE extract (CEW) contained maclurin 3-C-β-D-glucoside as common component.

Furthermore, the iriflophenone 3-C-(2,6-di-O-galloyl)-β-D-glucoside and 3,4-dihydroxybenzoic acid were only detected in the CEW extract. The phenolic compounds identified in the hydroalcoholic fraction (FEW) were similar to those detected in the pressurized hydroalcoholic extract (PEW). However, the previous extraction with pure CO_2 favors the concentration, particularly of iriflophenone derivatives and quercetin-arabinopyranoside. At the same time the content of gallic acid decreased, possibly because it was partially removed from the matrix during the first extraction with SC-CO_2 because this phenolic acid is slightly soluble in this solvent.

3.2. Anticarcinogenic activity in breast cancer cells

The anticarcinogenic activity of pressurized mango leaf extracts against breast cancer cells (MCF7 and MDA-MB-231) as well as non-tumorigenic mammary cells (MCF10A) was analyzed in this work. The predominant content of mangiferin and the presence of other polyphenols with antitumoral activities (gallic acid, quercetin and pentagalloylglucose) previously described in section 3.1 (**Table 1**) suggest the potential of pressurized mango leaf extracts as antitumoral agents in breast cancer therapy. In this sense, the effects of the extraction solvent (ethanol, ethanol-water 50:50, CO_2-ethanol-water 50:25:25 and a hydroalcoholic fraction previously extracted with SC-CO_2) and thus the phenolic profile of extracts on the antitumor activity of mango leaf extracts against breast cancer cells were evaluated. The results were analyzed in terms of the cellular cytotoxicity, the production of intracellular reactive oxygen species (ROS) and protection against oxidative damage.

3.2.1. Effect on cellular cytotoxicity

Uncontrolled cell division, together with suppressed apoptosis, is the primary key in the progression of human tumors. The cytotoxic activity of pressurized mango leaf extracts in human breast cancer cells (MDA-MB-231 and MCF7) and non-tumorigenic human mammary epithelial cells (MCF10A) at concentrations in the range 0.00001–0.1 mg/mL is shown in **Fig. 2**. It can be seen from this figure that cell survival was inhibited in breast cancer cells after exposure to

pressurized mango leaf extracts and this was dependent on the extraction solvent. The hydroalcoholic extract (PEW) and the hydroalcoholic fraction previously extracted with SC-CO_2 (FEW), which has similar phenolics profiles, showed greater cytotoxic activities in metastatic breast cancer MDA-MB-231 cells than the pressurized ethanolic extract (PET) and the ESE extract (CEW). The effect was more marked on using the fraction FEW, which was capable of reducing cell survival at values close to 40% after exposure to extract concentrations in the range 0.00001–0.0001 mg/mL (**Fig. 2.A**).

In contrast to the above, the pressurized ethanolic extract (PET) and the extract obtained with the enhanced mixture of CO_2-water-ethanol (CEW) showed cytotoxic effects on the minimally invasive MCF7 cells at concentrations in the range 0.00001–0.0001 mg/mL, with this effect also maintained by the CEW extract at concentration of 0.001 mg/mL. The other extracts analyzed (PEW and FEW) did not significantly modify the cellular viability of MCF7 breast cancer cells. However, the fraction FEW showed significant cell survival at the highest concentration applied (0.1 mg/mL) (**Fig. 2.B**).

The conventional aqueous mango bark extract Vimang has also shown cytotoxic activity in the metastatic breast cancer MDA-MB-231 cell line. However, higher concentrations of mango bark extract, i.e.; in the range 0.025–0.8 mg/mL, were required in order to inhibit cell viability in the MDA-MB-231 cell line in comparison with the lower doses used in the present study.[10] This finding suggests the potential antitumor activity of mango leaf extracts obtained by these novel techniques against breast cancer cells using small doses and considering the protective cytotoxic effect in normal mammary cells.

The cytotoxic effects that pressurized mango leaf extracts have on breast cancer cells can be attributed to the major polyphenols present in these extracts, which include mangiferin, gallic acid, quercetin glucosides, gallotannins and benzophenones. Mangiferin was the predominant phenolic compound present in mango leaf extracts obtained with ethanol (PET), ethanol-water (PEW) and CO_2-ethanol-water (CEW), and the second most abundant compound in the fraction FEW.

In a previous study mangiferin exhibited a non-cytotoxic effect in MDA-MB-231 on using concentrations between 0.025 and 0.8 mg/mL, although lower concentrations were not evaluated. Gallic acid showed dose-dependent cytotoxicity in MDA-MB-231 breast cancer cells with IC50 values of 0.01 mg/mL, suggesting that the low amounts of gallic acid present in mango extract are sufficient to induce significant anti-tumor effects.[10] In this sense, the results obtained for the fraction FEW are consistent with those described in the previous report. In fact, the results obtained in the present work show that cytotoxic activity on tumorigenic cells can be achieved using lower concentrations than reported previously by García-Rivera et al. (2011). FEW showed the highest cytotoxic effect in the highly aggressive MDA-MD-231 cell line, although it also presented the lowest content of gallic acid when compared with the other mango leaf extracts analyzed. However, the FEW mango leaf fraction did contain the highest concentration of other phenolic compounds such as iriflophenones and quercetin-arabinopyranoside.

The results obtained in the cytotoxicity study showed a relative relationship between the selectivity for a type of breast cancer cell and the phenolic profile of pressurized mango leaf extracts. Different minor components were detected in pressurized mango leaf extracts on changing the extraction solvent. These compounds could interfere with the antitumor activity and the specificity for a breast cancer cell type (estrogen and progesterone receptor-negative or -positive). As far as the selective cytotoxic effect of the extracts PET and CEW against MCF-7 breast cancer cells is concerned, it is important to note that these extracts contained the highest levels of gallotannins (tetragalloylglucose and pentagalloylglucose). Previous studies have shown that pentagalloylglucose inhibits breast cancer MCF-7 cell growth by suppression of the estrogen receptor function and it therefore may interfere with the cytotoxic effect of PET and PEW extracts against estrogen receptor-positive breast cancer cells.[17] Furthermore, this compound was not present in the fraction FEW, which had the lowest activity against the MCF-7 cell line. On the other hand, the extracts PET and CEW also had as common component the

benzophenone maclurin 3-C-βglucoside, which has shown biological activity based on the inhibitory effect on triglyceride accumulation.[15]
Other minor components could also interfere in the antitumor activity of pressurized mango leaf extracts. For example, the flavonoid quercetin showed a strong anti-proliferative effect in breast cancer cells independently of estrogen signaling, and regulatory capacity for apoptosis and anti-inflammation.[14,30-31] Other phenolic compounds such as benzophenones have also exhibited a regulatory capacity for pro-apoptotic genes in breast, prostate and colon cancers.[18]
Estrogen receptor-negative MDA-MB-231 cells were more susceptible to the cytotoxic effect of the pressurized hydroalcoholic extract (PEW) and the hydroalcoholic fraction previously extracted with SC-CO_2 (FEW), which had a similar phenolic profile to the other two extracts that had common minor components such as methyl gallate and homomangiferin. Nonetheless, further studies are required to define the influence of active compounds and synergistic effects involved in the antitumor activity of mango leaf extracts in breast cancer and the selectivity against aggressive or minimally invasive breast tumor cells. Previous studies have also indicated a negative correlation between the antiproliferative activities of mango extracts and the total phenolic content, thus suggesting that the antiproliferative and anticancer activities of mango extracts might depend on the combined effects of polyphenols and flavonoids present in the extracts.[32]
As far as the effect on non-tumorigenic cells is concerned, at the highest concentration (0.1 mg/ mL) all of the extracts were highly cytotoxic (**Fig. 2.C**). However, the extracts exhibited a cytotoxic effect on breast cancer cells but not in non-tumorigenic MCF10A cells at concentrations below 0.1 mg/mL. (**Fig. 2.C**). Only a significant decrease (less than 20%) in the number of living cells was observed in MCF10A cells at the lowest concentration applied (0.00001 mg/mL) on using the extracts CEW, PEW and FEW, and this effect was also observed at 0.0001 mg/mL on using the fraction FEW. In contrast, the pressurized ethanolic extract (PET) showed a slight proliferative effect at 0.001 and 0.01 mg/mL.

3.2.2. Effect on intracellular ROS levels

Reactive oxygen species (ROS) seem to be involved in cancer initiation since cancer cells use ROS-sensitive signaling pathways to launch proliferation, cell survival, glucose metabolism, invasion and other tumor progression mechanisms. The inhibition of ROS generation is therefore considered to be an antitumor effect of natural antioxidants and this may help to slow down tumor progression.[33-34]

In our previous work, the *in vitro* antioxidant activity of pressurized mango leaf extracts was evaluated by the DPPH assay. The results showed that mango leaf extracts presented a very potent antioxidant activity that was even higher than that of the recognized antioxidant α-tocopherol.[23] In the present work, the antioxidant activity was explored by intracellular ROS detection. Intracellular ROS levels in breast tumor cells and non-tumorigenic human breast epithelial cells treated with mango leaf extracts at concentrations in the range 0.00001–0.1 mg/mL were measured under basal conditions. The results for MDA-MB-231, MCF7 and MCF10A cells are shown in **Fig. 3**.

Treatment of cell lines with mango leaf extracts under basal conditions led to a significant decrease in intracellular ROS levels in MCF7 breast cancer cells (**Fig. 3B**). However, at the highest mango leaf extract concentration applied (0.1 mg/mL) all extracts seemed to be significant pro-oxidants for MCF7 and non-tumorigenic MCF10A cells (**Fig. 3C**). This trend was also observed in MDA-MB-231 cells although the results were statistically significant only for the pressurized hydroalcoholic extract (PEW) at 0.001 and 0.01 mg/mL (**Fig. 3A**).

The reduction in intracellular levels was dependent on the extraction solvent. The pressurized hydroalcoholic extract (PEW) showed the highest antioxidant effect in the three cell lines. A reduction of around 20% in ROS levels was observed only in the MDA-MB-231 cell line after treatment with the PEW extract at 0.001 and 0.01 mg/mL. In contrast, in MCF7 cells all of the different mango leaf extracts studied led to significant reductions in ROS levels (above 30%) on applying concentrations in the range 0.001–0.01 mg/mL, with this effect maintained in the pressurized ethanolic (PET) and hydroalcoholic

(PEW) extracts at a concentration of 0.0001 mg/mL and with PEW even at the lowest dose concentration applied (0.00001 mg/mL).
Finally, when MCF10A cells were treated with the pressurized hydroalcoholic extract (PEW) and the water-ethanol (FEW) fraction, the intracellular ROS levels at basal conditions decreased on increasing the concentration from 0.0001 to 0.01 mg/mL. As indicated previously, however, at 0.1 mg/mL all extracts seem to increase ROS levels in MCF10A and this is possibly due to an increase in basal oxidative stress, which may lead to a cell death. Therefore, the cytotoxicity of mango leaf extracts previously exhibited in MCF10A at the highest concentration applied (0.1 mg/mL) (**Fig. 2C**) could be promoted by severe oxidative stress induced by mango polyphenols.

3.2.3 Effect on protection after induced oxidative damage

In order to investigate the *in vitro* preventive effect of mango leaf extracts against H_2O_2 oxidative injury, H_2O_2 was added prior to fluorescence measurement on MCF7, MDA-MB-231 and MCF10A cell lines. Intracellular ROS levels were then measured in cells previously treated with mango leaf extracts at increasing concentrations from 0.00001 to 0.1 mg/mL and results are shown in **Fig. 4**. In terms of the oxidative stress stimulus, all pressurized extracts were able to reduce oxidative injury in both breast cancer cells (minimally invasive MCF7 and highly aggressive MDA-MB-231) and non-tumorigenic mammary epithelial cells (MCF10A) on using high dose concentrations (0.1 mg/mL). The effect was more marked in MDA-MB-231 and MCF10A cells, with a reduction of more than 60% oxidative injury with respect to the control.
In MDA-MB-231, the reduction in oxidative injury was observed as a significant decrease in a dose-dependent manner for all the extracts evaluated at concentrations of 0.01 and 0.1 mg/mL. The fraction FEW and the extract PEW also exhibited a significant decrease in intracellular ROS levels after the addition of H_2O_2 at a lower concentration of 0.001 mg/mL, but only the pressurized ethanolic extract (PEW) led to a significant reduction in oxidative injury at all the concentrations applied (**Fig. 4.A**).

However, after treatment with H_2O_2 the intracellular ROS levels in MCF7 decreased significantly only when cells were treated with the highest concentration applied (0.1 mg/mL) and also for the fraction FEW at 0.01 mg/mL (**Fig. 4.B**). Concentrations below 0.1 mg/mL did not reduce ROS levels after injury.

In contrast to the above, the use of the CEW extract at concentrations between 0.00001 and 0.001 mg/mL gave a statistically significant pro-oxidant effect in MCF7 cells. This effect was also observed in MDA-MB-231 cells but it was not statistically significant. This pro-oxidant effect could be useful in chemotherapies based on the increased oxidative stress above a toxic threshold level that kills cancer cells, while non-tumorigenic cells can bear these levels.[34] Accordingly, CEW extracts could be adjuvants for pro-oxidative cancer therapies because they increase ROS levels after H_2O_2-induced oxidative shock and they may promote the death of tumor cells without damage to epithelial cells.

In fact, on MCF10A cells all of the mango leaf extracts had a protective effect against H_2O_2-induced oxidative stress at the highest concentration applied (0.1 mg/mL) because intracellular ROS levels were greatly reduced (more than 40%) (**Fig. 4.C**). The pressurized ethanolic extract (PET) showed a significant antioxidant effect at all concentrations studied (0.00001–0.1 mg/mL), while the CEW and PEW extracts led to a slight decrease in intracellular ROS levels at 0.01 mg/mL and also at the highest concentration applied (0.1 mg/mL). In contrast, the hydroalcoholic fraction previously extracted with CO_2 (FEW) tended to increase ROS levels significantly at 0.0001 mg/mL.

The capacity of the pressurized mango leaf extracts to protect normal cells from oxidative damage can be explained by the high content of phenolic compounds such as phenolic acids, benzophenones, xanthones, flavonols and gallotannins. These polyphenols showed very potent scavenger activity and antioxidant properties in both in vitro and in vivo studies. In addition, mangiferin, one of the main components of mango leaves, has potent antioxidant properties that mainly involve metal chelation and scavenging activity.

According to the results analyzed, pressurized mango leaf extracts present antitumor activity and they can be considered as potential

agents for the treatment of breast cancer. Results suggest it would be possible to exploit the selective action of different mango leaf extracts depending on the characteristics of each tumor cell and with the advantage of not causing injury to normal mammary epithelial cells and providing protection from oxidative damage.

The extraction solvent play a key role in the production of mango leaf extracts by high-pressure techniques because different solvents lead to extracts with different functions and antitumoral effects. Extracts obtained by PLE using ethanol as the extraction solvent and by ESE using the mixture CO_2-ethanol-water 50:25:25 showed high cytotoxic effects on minimally invasive MCF7 breast tumor cells, whereas pressurized hydroalcoholic extracts – including the hydroalcoholic fraction previously extracted with SC-CO_2 – showed a marked antitumor activity against highly invasive MDA-MB-231 breast tumor cells.

Differences between the ROS levels were also observed. The hydroalcoholic extract presented the highest antioxidant effect in all of the cell lines investigated, whereas the ethanolic extract exhibited a protective effect against oxidative injury and the extract obtained using CO_2-ethanol-water showed a pro-oxidant effect in breast cancer cells.

In addition, although the potent antioxidant mangiferin was the predominant compound in the extracts, the results show that the selective inhibitory activity against hormone-receptor-positive (MCF7) and -negative (MDA-MB-231) breast cancer cells can be correlated with other minor polyphenols such as gallotannins, which have shown potential antitumoral activity against hormone-dependent breast cancer. However, more extensive studies on the chemical profiles and synergistic actions are necessary to understand the cytotoxic and antioxidant effects of the extracts obtained from this mango by-product.

ACKNOWLEDGES

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Farm 'La Mayora', Superior Centre of Scientific Research (CSIC), Malaga, Spain for supply the mango leaves.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURES

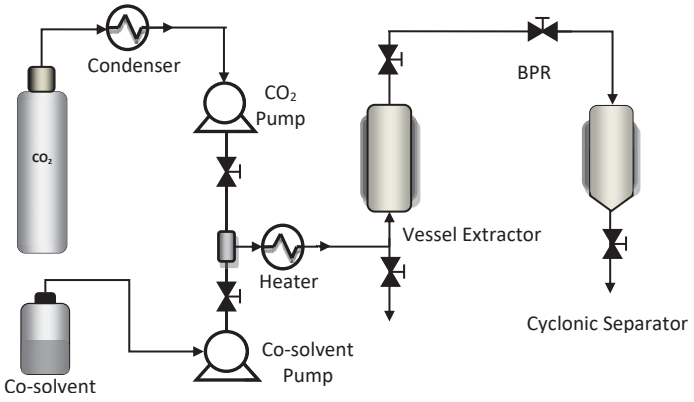
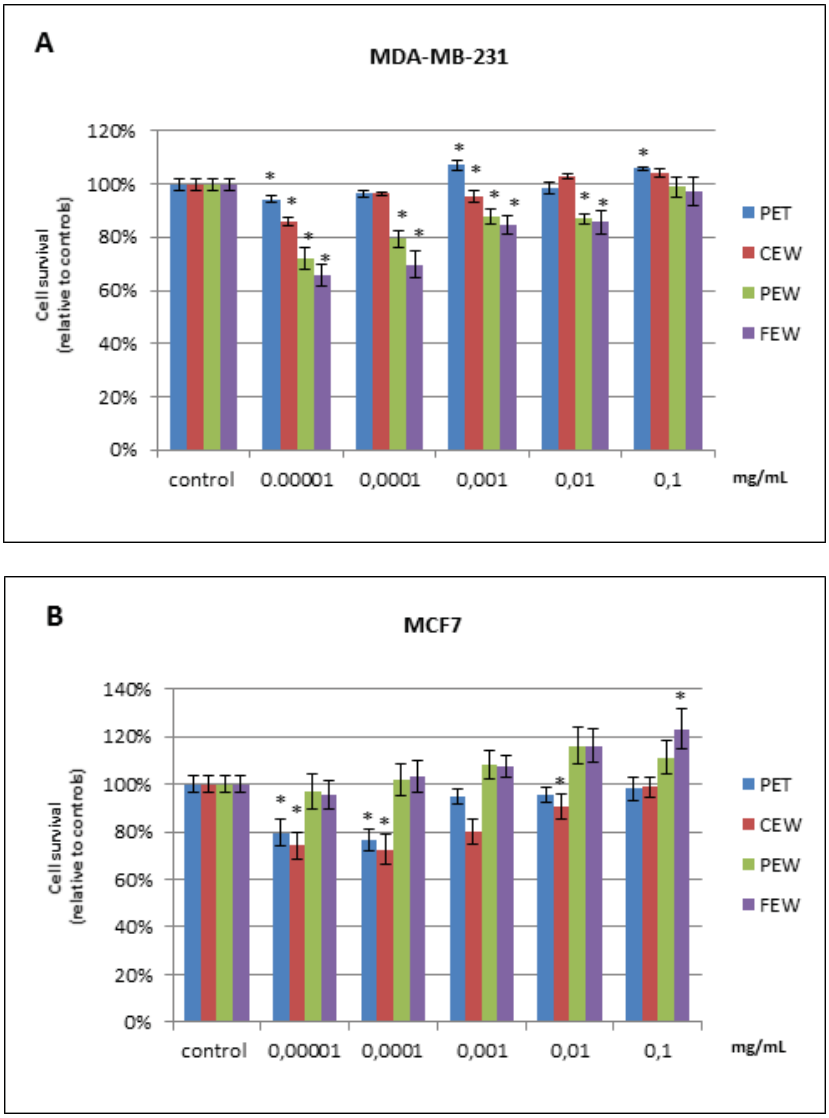


Figure 1. Schematic diagram of high pressure equipment

Figure 2. Cell survival of: A) MDA-MB-231 B) MCF7 and C) MCF10A cells measured by CellTiter Blue after 24 hours exposure to the extracts PET, CEW, PEW and FEW. Data are represented as the treatment average (±SEM) with respect to the control. *indicates statistically significant differences at $p < 0.05$.



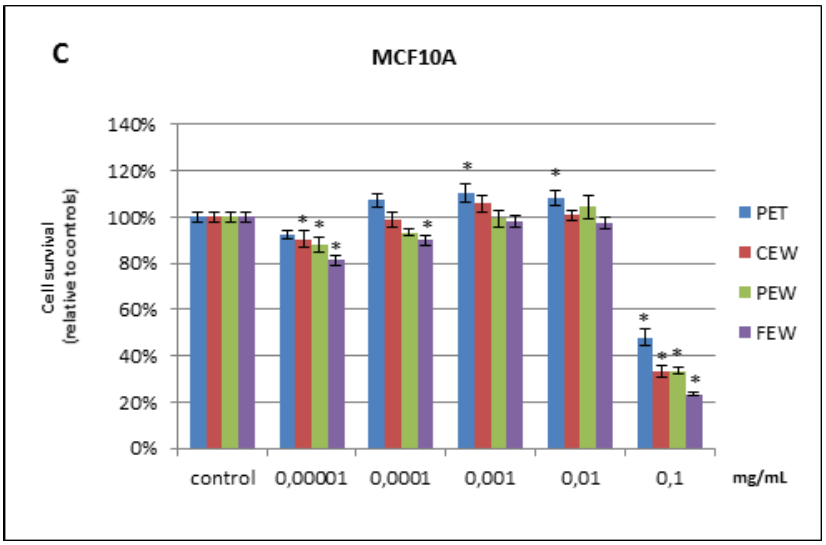
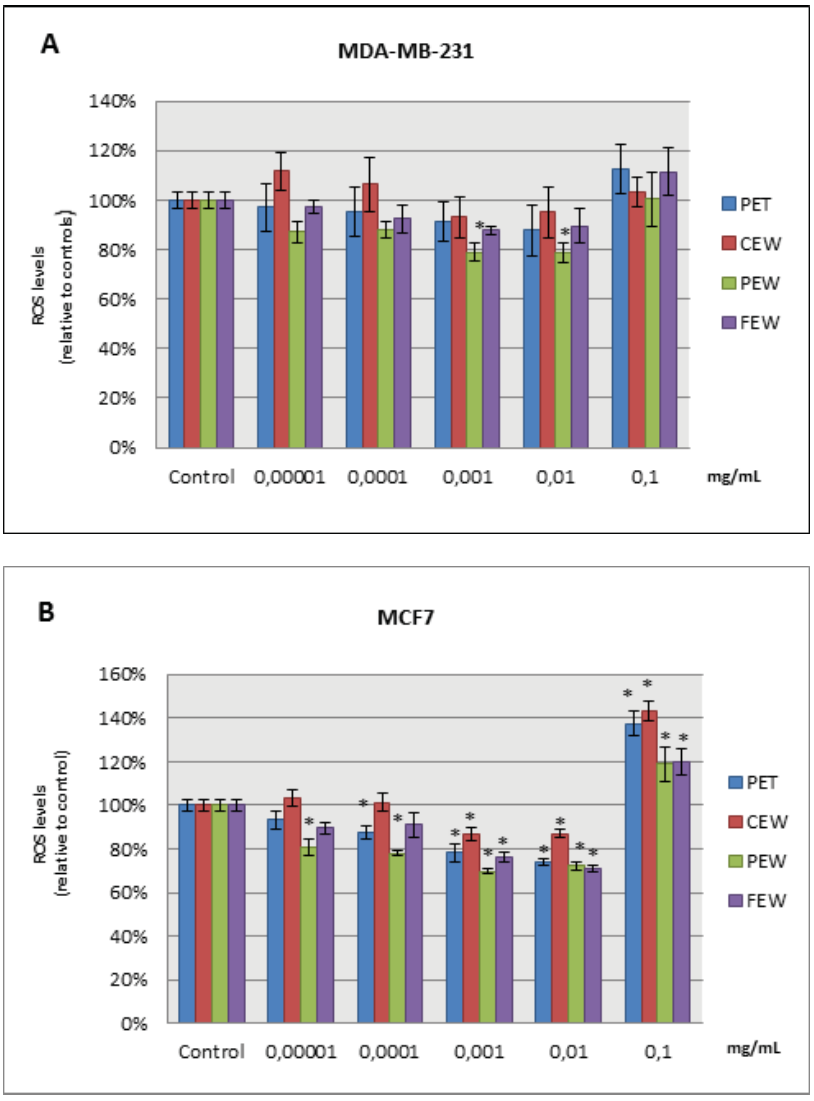

C
MCF10A
140%
120%
100%
80%
60%
40%
20%
0%
Cell survival (relative to controls)
control
0,00001
0,0001
0,001
0,01
0,1
mg/mL
PET
CEW
PEW
FEW

Figure 3. ROS levels under basal conditions in (A) MDA-MB-231, (B) MCF7 and (C) MCF10A after 24 hours of exposure to the extracts PET, CEW, PEW and FEW. Data are represented as the average (±SEM) with respect to a control without treatment of four independent assays carried out in triplicate. *represents statistically significant differences ($p<0.05$).



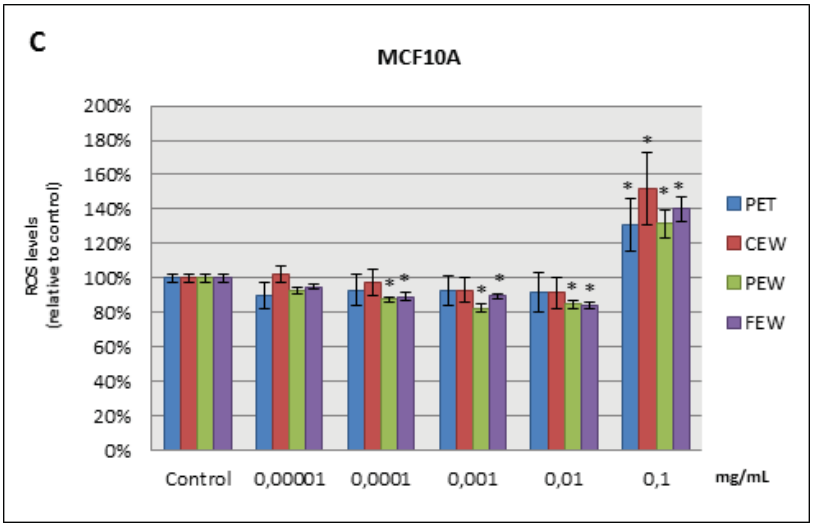
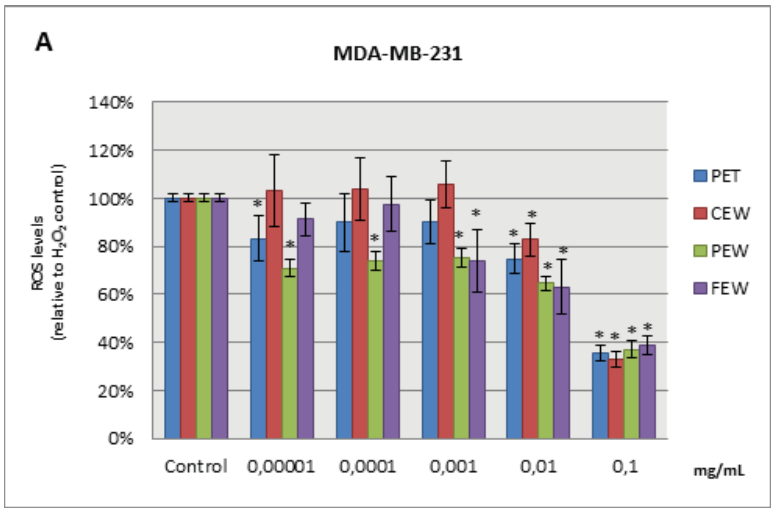
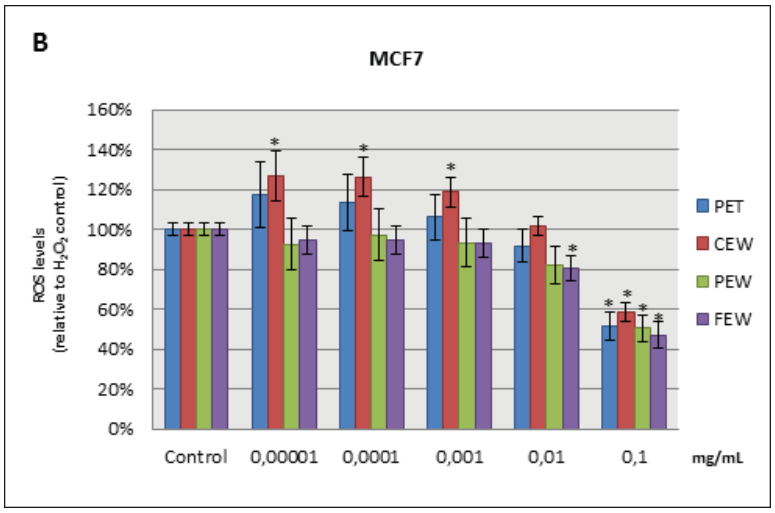
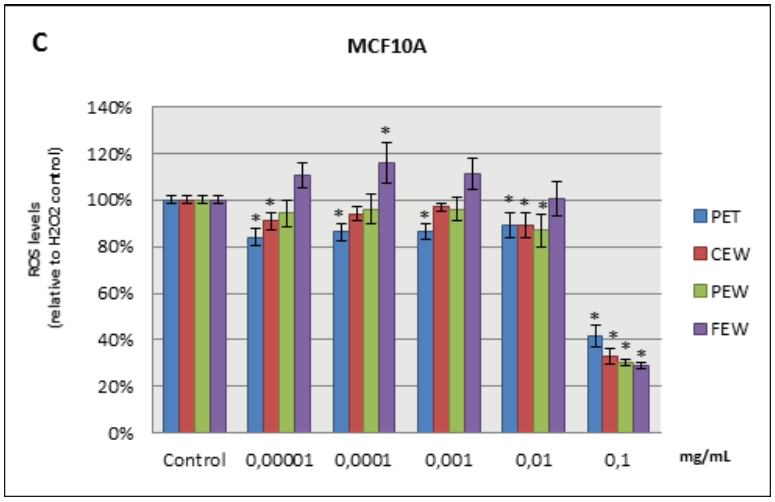

C
MCF10A
ROS levels
(relative to control)
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Control
0,00001
0,0001
0,001
0,01
0,1
mg/mL
PET
CEW
PEW
FEW

Figure 4. ROS levels in (A) MDA-MB-231, (B) MCF7 and (C) MCF10A cells after the addition of H_2O_2 measured with DCFH-DA after 24 hours exposure to the extracts PET, CEW, PEW and FEW. Data are represented as the average (±SEM) with respect to the control without treatment of four independents assays carried out in triplicate. *represents statistically significant differences ($p<0.05$).






C
MCF10A
ROS levels
(relative to H2O2 control)
140%
120%
100%
80%
60%
40%
20%
0%
Control
0,00001
0,0001
0,001
0,01
0,1
mg/mL
PET
CEW
PEW
FEW

TABLES

Table 1. Phenolic content of mango leaf extracts obtained on using ethanol (PEW), ethanol-water (PEW), water (SWE) and the hydroalcoholic fraction previously extracted with SC-CO_2 (FEW), expressed as g/100g dry extract and represented as the mean ± SD

Phenolic compounds	Mango leaf extracts			
	PET	**PEW**	**SWE**	**FEW**
water				
gallic acid	2.85 ± 0.01	3.09 ± 0.01	4.74 ± 0.05	3.26 ± 0.01
methyl gallate				
dihydroxibenzoic acid				
mangiferin	6.47 ± 0.01	7.60 ± 0.09	5.32 ± 0.07	6.20± 0.01
I 3-C-β-D-glucoside	6.30 ± 0.01	7.08 ± 0.04	5.16 ± 0.03	7.23 ± 0.01
I 3-C-(2-O-p-hydroxybenzoyl)-β-D-glucoside	5.32 ± 0.01	4.49 ± 0.02	4.00 ± 0.17	6.40 ± 0.01
I 3-C-(2-O-galloyl)-β-D-glucoside	1.17 ± 0.01	1.80 ± 0.55	1.82 ± 0.43	1.28 ± 0.01
Q 3-D-galactoside	1.03 ± 0.01	1.02 ± 0.15	1.16 ± 0.03	1.02 ± 0.01
Q 3-β-D-glucoside[a]	1.63 ± 0.01	1.61 ± 0.14	1.84 ± 0.22	1.64 ± 0.01
Q 3-O-xyloside	1.05 ± 0.01	1.03 ± 0.01	1.32 ± 0.09	1.08 ± 0.01
Q 3-O-α-L arabinopyranoside	1.04 ± 0.01	3.50 ± 0.11	1.51 ± 0.12	1.02 ± 0.01
Quercetin aglycone	nd	nd	nd	nd
tetra-O-galloyl-glucose	0.76 ± 0.01	0.67 ± 0.07	0.59 ± 0.04	0.84 ± 0.01
penta-O-galloyl-glucose	nd	nd	nd	nd
Q 3-D-galactoside	1.06 ± 0.01	1.31 ± 0.01	1.16 ± 0.03	1.02 ± 0.01
Q 3-β-D-glucoside[a]	2.16 ± 0.01	2.29 ± 0.01	1.79 ± 0.08	2.28 ± 0.35
Q 3-O-xyloside	1.28 ± 0.01	1.53 ± 0.01	0.54 ± 0.03	0.74 ± 0.10
Q 3-O-α-L arabinopyranoside	0.48 ± 0.01	0.56 ± 0.01	0.48 ± 0.03	0.79 ± 0.12
Quercetin aglycone	nd	nd	nd	nd
tetra-O-galloyl-glucose	0.31 ± 0.01	0.43 ± 0.01	0.60 ± 0.03	0.26 ± 0.02
penta-O-galloyl-glucose	0.21 ± 0.01	0.32 ± 0.01	0.23 ± 0.01	0.20 ± 0.02

Legend nd: not determinated or not detected, I: iriflophenone, Q: quercetin

REFERENCES

1. Sánchez-Quesada, C.; Lopez-Biedma, A.; Warleta, F.; Campos, M.; Beltrán, G.; Gaforio, J.J. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of *Olea europea*. *J. Agric. Food Chem.* **2013***, 61*, 12173-82.

2. López-Biedma A.; Sánchez-Quesada C.; Beltrán G.; Delgado-Rodríguez M.; Gaforio J.J. Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses. BMC *Complement. Altern. Med.* **2016a***, 16,* 350

3. Conklin, C.M.; Bechberger, J.F.; MacFabe, D.; Guthrie, N.; Kurowska, E.M.; Naus, C.C. Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells. *Carcinogenesis.* **2006***, 28*, 93-100.

4. Warleta F.; Campos M.; Allouche Y.; Sánchez-Quesada C.; Ruiz-Mora J.; Beltrán G.; Gaforio J.J. Squalene protects against oxidative DNA damage in MCF10A human mammaryepithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. *Food Chem. Toxicol.* **2010***, 48,* 1092–1100.

5. López-Biedma A.; Sánchez-Quesada C.; Delgado-Rodríguez M.; Gaforio J.J. The biological activities of natural lignans from olives and virgin olive oils: A review. *J. Funct. Foods.* **2016b***, 26*, 36-47.

6. Sánchez-Quesada, C.; Lopez-Biedma, A.; Gaforio, J.J. Oleanolic Acid, a Compound Present in Grapes and Olives, Protects against Genotoxicity in Human Mammary Epithelial Cells. *Molecules.* **2015a***, 20*, 13670-13688.

7. Sánchez-Quesada, C.; Lopez-Biedma, A.; Gaforio, J.J. The differential localization of a methyl group confers a different anti-breast cancer activity to two triterpenes present in olives. *Food Funct.* **2015b***, 6*, 249-256.

8. Vini R.; Sreeja S. *Punica granatum* and its therapeutic implications on breast carcinogenesis: A review. *Biofactors.* **2015***, 41,* 78-89

9. Yoshimi, N.; Matsunaga, K.; Katayama, M.; Yamada, Y.; Kuno, T.; Qiao, Z.; Hara, A.; Yamahara, J.; Mori, H. The inhibitory effects of mangiferin, a naturally occurring glucosylxanthone, in bowel carcinogenesis of male F344 rats. *Cancer Lett.* **2010***, 163,* 163-170.

10. García-Rivera, D.; Delgado, R.; Bougarne, N.; Haegeman, G.; Berghe, W.V. Gallic acid indanone and mangiferin xanthone are strong determinants of immunosuppressive anti-tumour effects of *Mangifera indica L.* bark in MDA-MB231 breast cancer cells. *Cancer Lett.* **2011***, 305*, 21-31.

11. Li H.; Huang J.; Yang B.; Xiang T.; Yin X.; Peng W.; Cheng W.; Wan J.; Luo F.; Li H.; Ren G. Mangiferin exerts antitumor activity in breast cancer cells by regulating matrix metalloproteinases, epithelial to mesenchymal transition, and β-catenin signaling pathway. *Toxicol. Appl. Pharm.* **2013***, 272*, 180–190.

12. Jung, J.S.; Jung, K.; Kim, D.H.; Kim, H.S. Selective inhibition of MMP-9 gene expression by mangiferin in PMA-stimulated human astroglioma cells: Involvement of PI3K/Akt and MAPK signaling pathways. *Pharmacol. Res.* **2012***, 66,* 95–103.

13. Rivera-Rivera, A.; Castillo-Pichardo, L.; Gerena, Y.; Dharmawardhane, S. Anti-Breast Cancer Potential of Quercetin via the Akt/AMPK/Mammalian Target of Rapamycin (mTOR) Signaling Cascade. *PLoS One.* **2016***, 11,* e0157251. doi: 10.1371/journal.pone.0157251.

14. Chien, S.Y.; Wu, Y.C.; Chung, J.G.; Yang, J.S.; Lu, H.F.; Tsou, M.F.; Wood, W.G.; Kuo, S.J.; Chen, D.R. Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. *Hum Ex Toxicol.* **2009***, 8*, 493-503.

15. Zhang J.; Li L.; Kim S.H.; Hagerman A.E.; Lü J. Anti-cancer, Anti-diabetic and other pharmacologic and biological activities of Penta-Galloyl-Glucose. *Pharm. Res.* **2009***, 26,* 2066-2080.

16. Zhang H.; Zhang M.; Yu L.; Zhao Y.; He N.; Yang X. (2012). Antitumor activities of quercetin and quercetin-5',8-disulfonate in human

colon and breast cancer cell lines. *Food Chem. Toxicol* . **2012**, *50,* 1589-99.

17. Hua, K.T.; Way, T.D.; Lin, J.K. Pentagalloylglucose inhibits estrogen receptor alpha by lysosome-dependent depletion and modulates ErbB/PI3K/Akt pathway in human breast cancer MCF-7 cells. *Mol. Carcinog.* **2006**, *45*, 551-560.

18. Noratto, G.D.; Bertoldi, M.C.; Krenek, K.; Talcott, S.T.; Stringheta, P.C.; Mertens-Talcott, S.U. Anticarcinogenic Effects of Polyphenolics from Mango (*Mangifera indica*) Varieties. *J. Agric. Food Chem.* **2010**, *58*, 4104–4112.

19. Shaban, N.Z.; Hegazy, W.A.; Abdel-Rahman, S.M.; Awed, O.M.; Khalil, S.A. Potential effect of *Olea europea* leaves, *Sonchus oleraceus* leaves and *Mangifera indica* peel extracts on aromatase activity in human placental microsomes and CYP19A1 expression in MCF-7 cell line: Comparative study. *Cell Mol. Biol. (Noisy-le-grand).* **2016**, *62*, 11-9.

20. Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; Rodríguez, M.; Martínez de la Ossa, E. Extraction of antioxidant compounds from different varieties of *Mangifera indica* leaves using green technologies. *J. Supercrit. Fluids.* **2012**, *72*, 168–175

21. Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; Martínez de la Ossa, E. Potential use of mango leaves extracts obtained by high pressure technologies in cosmetic, pharmaceutics and food industries. *Chem. Eng. Trans.* **2013**, *32*, 1147–1152, http://dx.doi.org/10.3303/CET1332192.

22. Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; Martínez de la Ossa, E. Fractionation of *Mangifera indica Linn* polyphenols by reverse phase supercritical fluid chromatography (RP-SFC) at pilot plant scale. *J. Supercritic. Fluids.* **2014**, *95*, 444–456

23. Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; Martínez de la Ossa, E. Use of high pressure techniques to produce *Mangifera indica L.* leaf extracts enriched in potent antioxidant phenolic compounds. *Innov. Food Sci. Emerg.* **2015**, *29*, 94–106

24. Fernández-Ponce, M.T.; Parjikolaei, B.R.; Lari, H.N.; Casas, L.; Mantell, C.; Martínez de la Ossa, E. Pilot-plant scale extraction of

phenolic compounds from mango leaves using different green techniques: Kinetic and scale up study. *Chem. Eng. J.* **2016***, 299,* 420-430.

25. García-Mendoza, M.P.; Paula, J.T.; Paviani, L.C.; Cabral, F.A.; Martínez-Correa, H.A. Extracts from mango peel by-product obtained by supercritical CO_2 and pressurized solvent processes. *LWT - Food Sci. Technol.* **2015***, 62*, 131-137.

26. Infante-García, C.; Ramos-Rodríguez, J.J.; Delgado-Olmos, I.; Gamero-Carrasco, C.; Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; García-Alloza, M. Long-Term Mangiferin Extract Treatment Improves Central Pathology and Cognitive Deficits in APP/PS1 Mice. *Mol. Neurobiol.* **2016a**, DOI 10.1007/s12035-016-0015-z

27. Infante-García, C.; Ramos-Rodríguez, J.J.; Marin-Zambrana, Y.; Fernández-Ponce, M.T.; Casas, L.; Mantell, C.; García-Alloza, M. Mango leaf extract improves central pathology and cognitive impairment in a type 2 diabetes mouse model. *Brain Pathol.* **2016**, DOI 10.1111/bpa.12433.

28. Mohan, C.G.; Deepak, M.; Viswanatha, G.L.; Savinay, G.; Hanumantharaju, V.; Rajendra, C.E.; Halemani, P.D. Anti-oxidant and Anti-inflammatory of leaf extracts and fractions of *Mangifera indica. Asian Pac. J. Trop. Med.* **2013***,* 311-314.

29. Lee, D.H.; Lim, B.S.; Lee, Y.K.; Yang, H.C. Effects of hydrogen peroxide (H_2O_2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biol. Toxicol.* **2006***, 22*, 39-46.

30. Choi, E.J.; Bae, S.M.; Ahn, W.S. Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. *Arch Pharm Res.* **2008***, 10*, 1281-5.

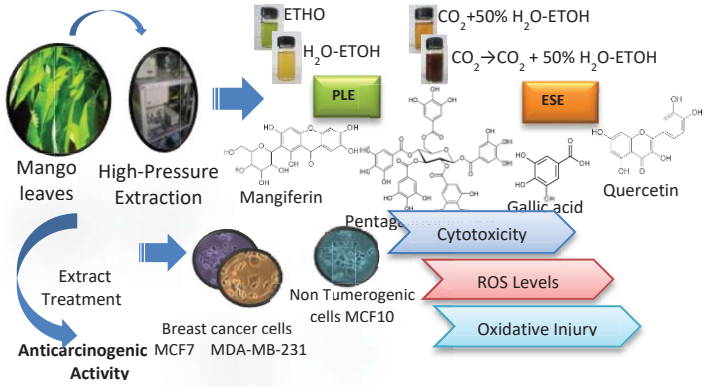
31. Chou, C.C.; Yang, J.S.; Lu, H.F.; Ip, S.W.; Lo, C.; Wu, C.C.; Lin, J.P.; Tang, N.Y.; Chung, J.G.; Chou, M.J.; Teng, Y.H.; Chen, D.R. Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. *Arch Pharm Res.* **2010***, 33,* 1181-91.

32. Kim, H.; Moon, J.Y.; Kim, H.; Lee, D.S.; Cho, M.; Choi, H.K.; Kim, Y.S.; Mosaddik, A.; Cho, S.K. Antioxidant and antiproliferative activities of mango (*Mangifera indica L.*) flesh and peel. *Food Chem.* **2010**, *121*, 429–436.

33. Liou, G.Y.; Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **2010**, *44*, doi:10.3109/10715761003667554.

34. Nogueira, V.; Hay, N. Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. *Clin. Cancer Res.* **2013**, *19*, 4309-4314.

GRAPHICAL ABSTRACT



Anexo II

Artículo de divulgación

Sánchez-Quesada, C., **López-Biedma, A.**, Gaforio, J.J. Natural protection against cancer by minor compounds found in virgin olive oil.. *Jaen Journal on Approximation.* 2015.

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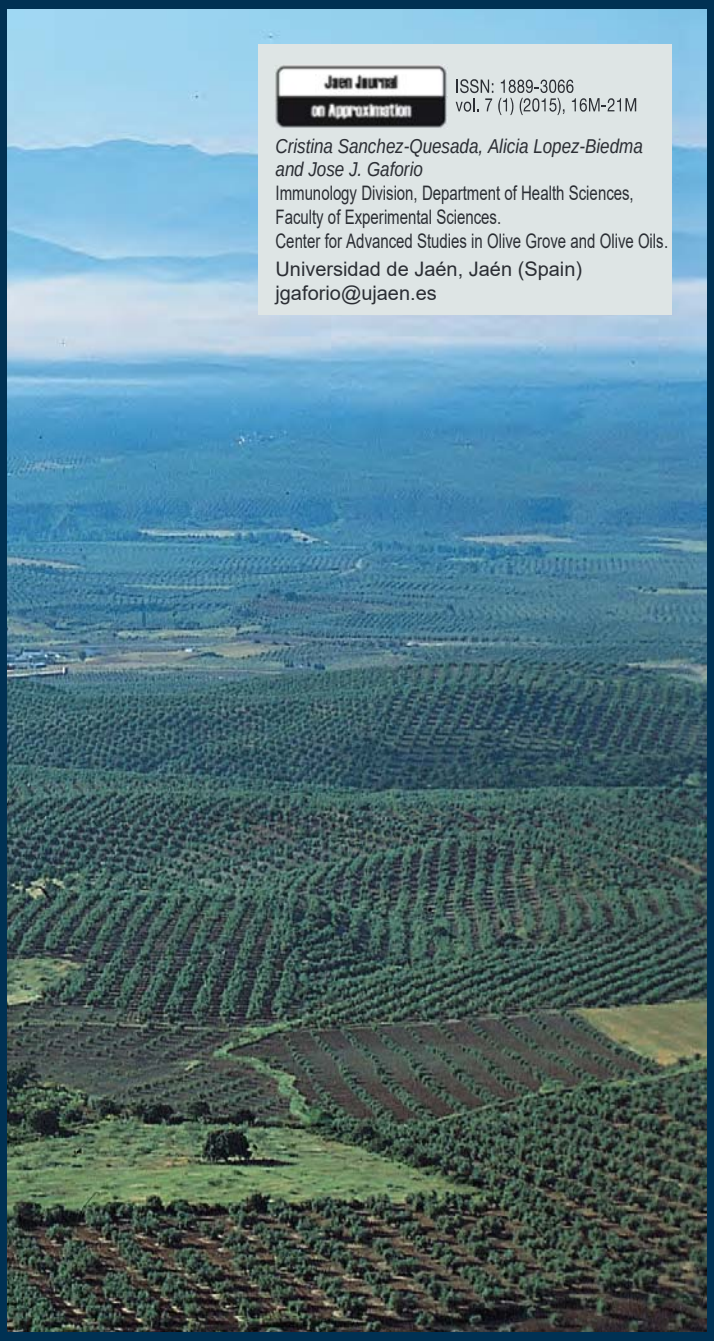
Natural protection against cancer by minor compounds found in virgin olive oil



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ABSTRACT

Virgin olive oil is the main fat used in the Mediterranean diet. This natural fruit juice contents about 230 different compounds in its minor fraction able to exert beneficial properties in several diseases. Squalene, oleanolic acid, maslinic acid, uvaol and erytrodiol are five of the most representative minor compounds found in virgin olive oil. All of these virgin olive oil compounds have antitumoral effects in different types of cancer, even more they have a natural preventive role in these types of cancer. All the studies remark their potential roles like chemopreventives and even chemotherapeutics in several cases. In conclusion, several beneficial antitumoral properties of virgin olive oil can be explained by its composition, and specifically by the presence of these five compounds in virgin olive oil.

1. Virgin olive oil

The Mediterranean diet has been described to prevent different illness ([45]) and promote human health benefits ([11, 33, 36]).

The most of these benefits could be attributed to Virgin Olive Oil (VOO) which is the main fat source of this kind of diet. Among others, VOO has been described to protect against cardiovascular diseases ([26, 29]), has anti-inflammatory effects ([17]) and promotes apoptosis in different kind of cancers ([12, 2, 28, 27, 34]). It is believed

that these healthy effects are due to minor compounds present in VOO. VOO 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 is an amount of minor compounds (among 230 different compounds). These minor compounds are being studied for the antitumor effects and preventive actions ([4,5, 32, 44, 3]).

1.1 Minor compounds of virgin olive oil

Among the minor compounds of VOO, there are different groups of natural compounds distinct each other for its chemical structure. They can also be used as effective finger prints (i.e. biomarkers) to evaluate quality and authentication of the olive oils ([1]). Polyphenols, tocopherols, sterols, carotenes, triterpenes and hydrocarbons are some of the groups that we can find in VOO.

There is a major hydrocarbon present at high concentration in shark liver and VOO, squalene (SQ). This compound is an intermediate metabolite in cholesterol metabolism.

SQ, together with the main triterpenes are the most representative compounds of VOO. Oleanolic acid (OA) and maslinic acid (MA) are triterpenic acids present in VOO and uvaol and erytrodiol are triterpenics alcohols. These triterpenes are present in the leaves and skin of olives ([1]).

1.2 Characterization of squalene, oleanolic acid, maslinic acid, uvaol and erytrodiol.

Squalene

Squalene (SQ) is synthetized by squalene synthase, an enzyme that condenses two molecules of farnesyl pyrophosphate with reduction by NADPH to form squalene (Figure 1).

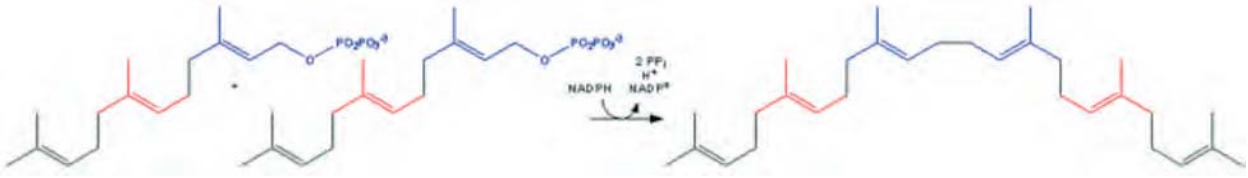

Figure 1. Synthesis of squalene by squalene synthase.

This molecule is a natural 30-carbon organic compound acting as biochemical intermediate in animals (it is the precursor of lanosterol and cholesterol) and plants (being the precursor of stigmasterol). SQ is a hydrocarbon and a triterpene, and is a natural and vital part of the synthesis of all plant and animal sterols, including cholesterol, steroid hormones and vitamin D in the human body ([42]).

In VOO, SQ is the major hydrocarbon (more than 90%), with content ranging from 0,8 to 13 g/kg ([47]).

Oleanolic and maslinic acid

Oleanolic (OA) and maslinic acid (MA) are two hydroxyl pentacyclic triterpene acids differentiated by one vicinal hydroxyl groups at the C-2 position (Figure 2).

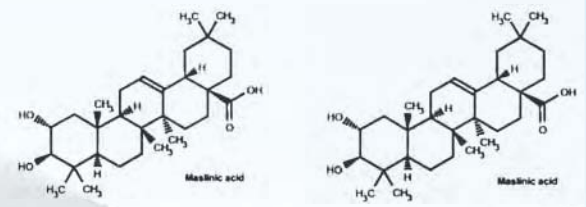

Figure 2. Chemical structure of OA and MA.

These triterpenes are found in olive skin and the leaves of olive tree (Olea Europaea). The Picual variety shows the highest content of this two triterpenic acids with quantities around 110 mg/kg ([1]).

Uvaol and erytrodiol

These triterpenic alcohols are two molecules synthetized in olive tree also, but very little is known about their activity in the human body. However, both possess interesting and useful activities in several diseases. Uvaol (UV) and erytrodiol (ER) differ in a functional group at the C-17 position, which is located in another carbon in uvaol molecule (Figure 3).

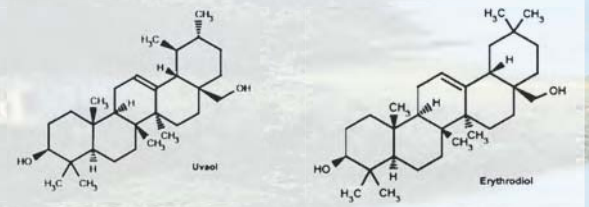

Figure 3. Chemical structure of UV and ER.

Both compounds appear at minor concentrations that triterpenic acids in olives and leaf of the tree ([1]).

Squalene is the precursor of the four triterpenes in the formation of leaves and fruits in the olive tree (Figure 4).

The concentration of all the minor compound fraction will depend on the genetic factors, handing of olive oil and in summary, in the quality of the virgin olive oil ([41]).

2. Bioactivity of minor compounds of virgin olive oil (SQ, OA, MA, UV and ER) in cancer

2.1 Squalene

The major hydrocarbon present in VOO has preventive effects in different illness, like Parkinson disease ([18]) or cardiovascular diseases ([14,37, 3, 6, 15, 16, 24, 30, 37]). But its effects in cancer have been more associated to the preventive that to the antitumoral role. Indeed, some new strategies in cancer are to test squalene derivatives and improve their effectiveness in certain types of cancer ([8, 42, 46]).

SQ is very well-known for its preventive role against several human diseases ([7, 20, 23, 25]) but only a few articles

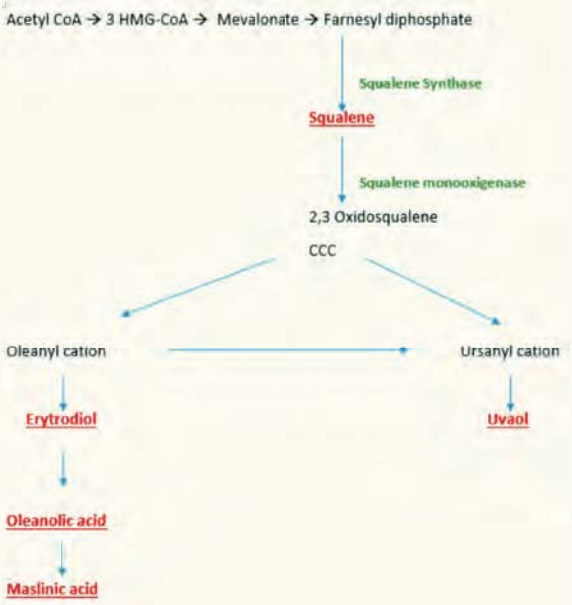

Figure 4. Resume of the biosynthesis taking place in olives and leaf of Olea europaea. "CCC" refers to triple chair form of the molecule.

describe its antitumoral effects ([31, 43]). But the preventive effects are very large, making this compound a good natural option for preventing diverse kinds of cancer diseases ([42]).

SQ has chemopreventive effects in breast cancer in vitro ([47]), on a neuroblastoma model in vitro ([10]), it protects bone marrow progenitors ([9]) and prevents skin, colon and lung cancer ([31, 35, 43]). Moreover, SQ is able to inhibit aberrant hyperproliferation, an event that precedes mammary tumourigenesis in vivo ([19]).

These chemopreventive effects appear to be due to an antioxidant mechanism that this compound promotes inside the cell ([47, 22]), that prevent it from an increase of reactive oxygen species (ROS), that otherwise are responsible of certain kind of carcinogenesis ([21]).

2.2 Oleanolic acid

OA has been studied in different types of cancer cells. And one common effect that oleanolic acid exhibits is its proapoptotic and antiproliferative actions in hepatocellular carcinoma cells, pancreatic carcinoma, non-small-cell lung cancer, lung adenocarcinoma, melanoma cells, breast cancer and colon cancer ([41]). This compound bases its action in the activation cascade of caspases, specifically caspase-3 and caspase-8. OA appears to induce cell cycle arrest in tumoral cells and to control angiogenesis ([41]), a main step in tumor development.

There is no evidence about their preventive role, except for their preventive role in breast human cells in vitro ([40]), but all the scientific works found suggest that this compound is able to have antitumoral effects due to its antioxidant action. While other compounds are not capable of promoting an antioxidant microenvironment for the cell, OA is ([41]).

2.3 Maslinic acid

Scientific studies describe the chemopreventive potential of MA in colorectal cancer in vitro and in vivo and the interesting potential that it has in targeting pro-inflammatory pathways as natural cancer prevention ([41]). But this compound has antimetastatic activity in the development of the different tumors (i.e. prostate cancer cells), activity not described yet in any minor olive oil compound ([34]).

MA also induces apoptosis through caspases pathway in different types of cancer cells as murine melanoma, human colon cancer or salivary gland adenoid cystic carcinoma ([41]).

But like it was mentioned before, MA has natural chempreventive effects through regulation of pro-inflammatory pathways ([39]). MA is able to enhance pro-inflammatory response in human body, to prevent carcinogenesis and even more, MA could intensify inflammatory human response in the early stages of tumor development.

2.4 Uvaol

UV is a triterpenic alcohol not as studied as the triterpenic acids, but with interesting properties. UV inhibits proliferation of several cancer cells of multiple origin like leukemic cells ([13]). A recent work describes the protective role of uvaol in human mammary cells compared to erytrodiol ([38]).

2.5 Erytrodiol

Unless there is no evidence of preventive role of ER in any human cell, ER exerts strong antiproliferative effects in several types of cancer such as skin tumor, breast cancer cells, astrocytoma cells and lymphoma cells ([41]).

Unless ER only differs in the location of a methyl group respect to UV, this distinction confers an antitumoral effect in metastatic breast cancer cells, promoting DNA damage in them ([38]).

3. Conclusion

Numerous scientific evidences support the beneficial properties of squalene, maslinic acid, oleanolic acid, erytrodiol and uvaol. Since all of them are present in virgin olive oils, they could be responsible, at least partially, of health claims attributed to the consumption of these oils. Overall, the available scientific evidence suggests that consumption of virgin olive oils rich in these minor compounds, can contribute to the prevention of certain cancers such as breast cancer or colon cancer.

Bibliography

[1] Allouche, Y. et al. Journal of Agricultural and Food Chemistry 57 (9): 3604-3610; 2009.

[2] Allouche, Y. et al. Journal of Agricultural and Food Chemistry 59 (1): 121-130; 2011.

[3] Bullon, P. et al. Food and Chemical Toxicology 47 (9): 2327-2331; 2009.

[4] Cardeno, A. et al. Food Chemistry 161: 353-360; 2014a.

[5] Cardeno, A.et al. Food & Function. 5 (6): 1270-1277; 2014b.

[6] Chan, P. et al. Journal of Clinical Pharmacology 36 (5): 422-427; 1996.

[7] Cho, S. et al. Clinical and Experimental Dermatology 34 (4): 500-508; 2009.

[8] Cosco, D. et al. International Journal of Nanomedicine 7: 2535-2546.; 2012.

[9] Das, B. et al. Neoplasia 10 (10): 1105-1119; 2008.

[10] Das, B. et al. European Journal of Cancer 39 (17): 2556-2565; 2003.

[11] De Filippis, F. et al. Gut doi: 10.1136/gutjnl-2015-309957; 2015.

[12] Escrich, E. et al. Public Health Nutrition 14 (12A): 2323-2332; 2011.

[13] Es-Saady, D. et al. Mediators of Inflammation 3 (3): 181-184; 1994.

[14] Farvin, K. H. et al. Journal of Medicinal Food 9 (4): 531-536; 2006.

[15] Granados-Principal, S. et al. Molecular Nutrition and Food Research 56 (5): 733-740; 2012.

[16] Guillén, N. et al. Atherosclerosis 197 (1): 72-83; 2008.

[17] Huang, L. et al. European Journal of Pharmacology 672 (1-3): 169-174; 2011.

[18] Kabuto, H. et al. Journal of Oleo Science 62 (1): 21-28; 2013.

[19] Katdare, M. et al. Cancer Letters 111 (1-2): 141-147; 1997.

[20] Kelly, G. S. Alternative Medicine Review 4 (1): 29-36; 1999.

[21] Klaunig, J. E. and L. M. Kamendulis. Annual Review of Pharmacology and Toxicology 44: 239-267; 2004.

[22] Kohno, Y. Biochimica et Biophysica Acta 1256 (1): 52-56; 1995.

[23] Kostyuk, V. et al. PLoS One 7 (8): e44472; 2012.

[24] Kritchevsky, D. et al. Archives of Biochemistry and Biophysics 44 (1): 241; 1953.

[25] Lindholm, J. S. and D. T. Downing. Lipids 15 (12): 1062-1063; 1980.

[26] Lou-Bonafonte, J. M. Current Vascular Pharmacology 10 (4): 392-409; 2012.

[27] Lucio, K. A. et al. PloS One 6 (12): e28596; 2011.

[28] Martin, R. et al. PloS One 4 (6): e5975; 2009.

[29] Martinez-Gonzalez, M. A. et al. Circulation 130 (1): 18-26; 2014.

[30] Motawi, T. et al. Food and Chemical Toxicology 48 (8-9): 2326-2336; 2010.

[31] Murakoshi, M. et al. International Journal of Cancer 52 (6): 950-952; 1992.

[32] Muscoli, C. et al. Journal of Biological Regulators and Homeostatic Agents 28 (1): 105-116; 2014.

[33] Noites, A. et al. Portuguese Journal of Cardiology 34 (11): 655-664; 2015.

[34] Park, S. Y. et al. The British Journal of Nutrition 109 (2): 210-222; 2013.

[35] Rao, C. V. et al. Carcinogenesis 19 (2): 287-290; 1998.

[36] Rotelli, M. T. et al. Surgical Oncology 24 (3): 145-152; 2015.

[37] Sabeena Farvin, K. H. et al. Pharmacological Research 50 (3): 231-236; 2004a.

[38] Sanchez-Quesada, C. et al. Food & Function 6 (1): 248-255; 2015a.

[39] Sanchez-Quesada, C. et al. Evidence-Based Complementary and Alternative Medicine: 654721; 2015b.

[40] Sanchez-Quesada, C. et al. Molecules 20 (8): 13670-13688; 2015c.

[41] Sanchez-Quesada, C. et al. Journal of Agricultural and Food Chemistry 61 (50): 12173-12182; 2013.

[42] Smith, T. J. Expert Opinion on Investigational Drugs 9 (8): 1841-1848; 2000.

[43] Smith, T. J. et al. Carcinogenesis 19 (4): 703-706; 1998.

[44] Stiti, N. et al. In Olives and Olive Oil in Health and Disease Prevention, edited by Victor R. Preedy and Ronald Ross Watson, 211-218; 2010.

[45] Toledo, E. et al. JAMA Internal Medicine 175 (11): 1752-1760; 2015.

[46] Vera, B., A. D. et al. European Journal of Organic Chemistry 2009 (31): 5327-5336; 2009.

[47] Warleta, F. et al. Food and Chemical Toxicology 48 (4): 1092-1100; 2010.

Anexo III

Capítulos de libros

"Molecular aspects of squalene and implications for olive oil and the Mediterranean diet". José J. Gaforio, Cristina Sánchez-Quesada, Alicia López-Biedma, Mª del Carmen Ramírez-Tortosa y Fernando Warleta.

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An Evidence-Based Approach

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Ronald Ross Watson

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Edited by

Victor R. Preedy
King's College London, London, UK

Ronald Ross Watson
University of Arizona, Tucson, AZ, USA

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Chapter 26

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

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ABBREVIATIONS

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

MEDITERRANEAN DIET

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

VIRGIN OLIVE OIL

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

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

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

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

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

SQUALENE IN VIRGIN OLIVE OIL

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

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

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

SQUALENE IN HUMANS

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

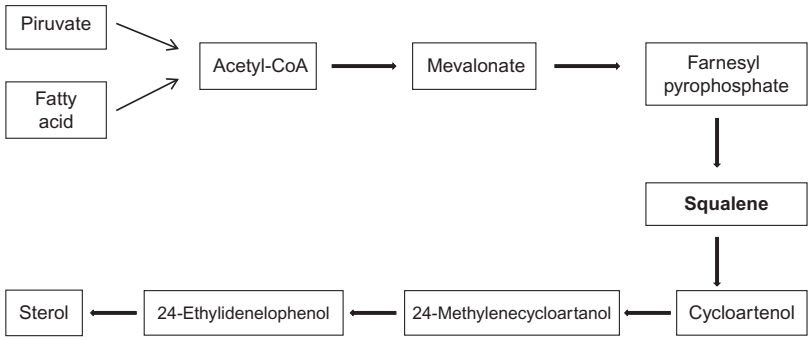

FIGURE 1 Sterols synthesis pathway in olive fruit.

TABLE 1 Composition of Sebum in Humans

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

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

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

BIOLOGICAL ACTIVITIES OF SQUALENE

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

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

Skin and Eyes

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

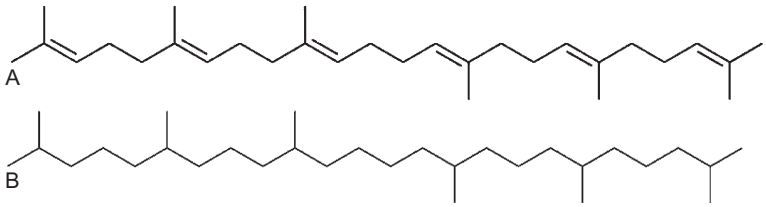

FIGURE 2 Chemical structure of squalene (a) and squalane (b).

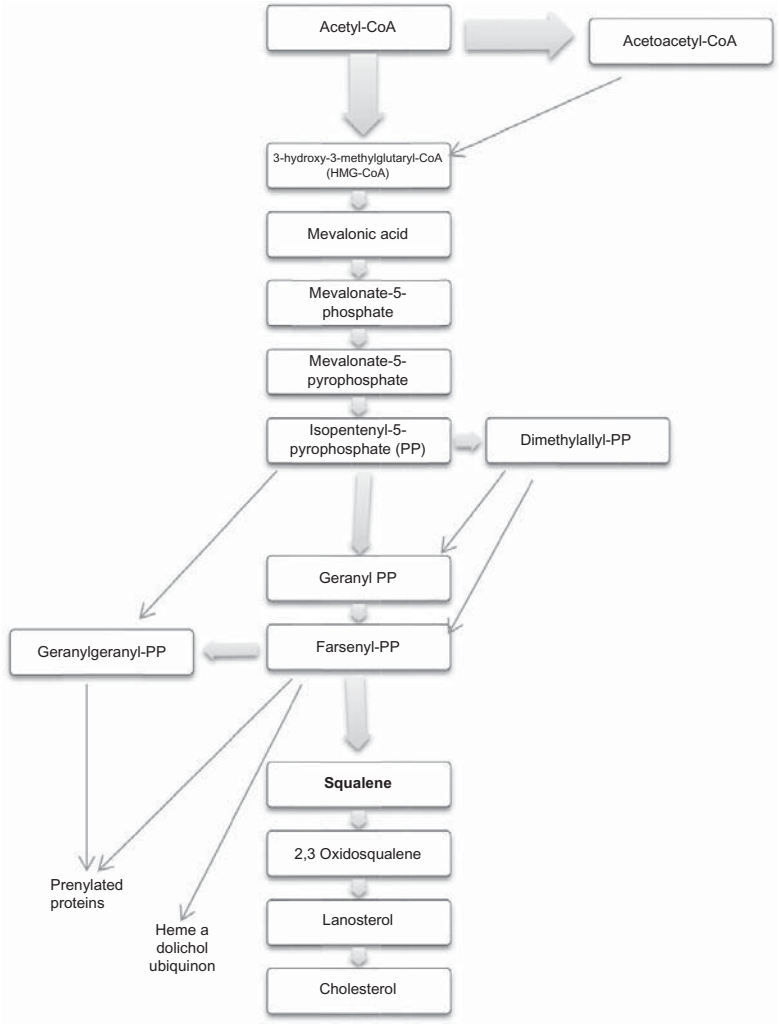

FIGURE 3 Cholesterol metabolic pathway. The schematic representation of the cholesterol biosynthetic pathway includes the squalene, a precursor of cholesterol.

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

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

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

Serum Cholesterol Concentration

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

Cardiovascular Disease

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

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

Cancer

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

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

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

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

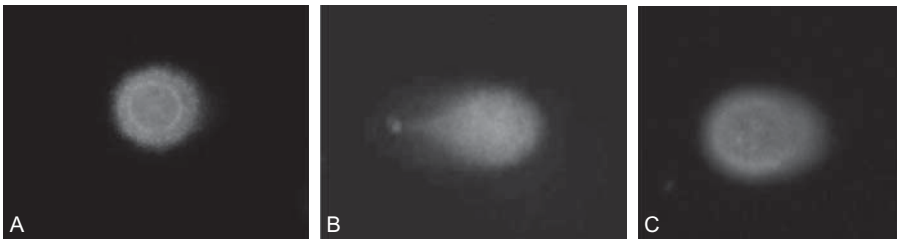

FIGURE 4 Representative images of Comet assay analysis of MCF10A (human mammary epithelial cells) untreated cell (a); 10 min H_2O_2 exposed cell (b) and; 10 min H_2O_2 exposed after 24 h Squalene 200 μM pretreated cell (c). (a) Cell shows a circular form indicating absence of DNA damage. (b) Cell exhibits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

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

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

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

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

SUMMARY POINTS

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

TABLE 2 Summary of the Biological Activities of Squalene Described

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

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

REFERENCES

[1] Willett WC. The Mediterranean diet: science and practice. Public Health Nutr 2006;9(1A):105–10.
[2] Sofi F, Abbate R, Gensini GF, Casini A. Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. Am J Clin Nutr 2010;92(5):1189–96.
[3] Caramia G, Gori A, Valli E, Cerretani L. Virgin olive oil in preventive medicine: from legend to epigenetics. Eur J Lipid Sci Technol 2012;114(4):375–88.
[4] Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Arós F, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med 2013;368(14):1279–90.
[5] Allouche Y, Jimenez A, Gaforio JJ, Uceda M, Beltran G. How heating affects extra virgin olive oil quality indexes and chemical composition. J Agric Food Chem 2007;55(23):9646–54.
[6] Liu GCK, Ahrens Jr EH, Schreibman PH, Crouse JR. Measurement of squalene in human tissues and plasma: validation and application. J Lipid Res 1976;17(1):38–45.
[7] Hrncirik K, Fritsche S. Relation between the endogenous antioxidant system and the quality of extra virgin olive oil under accelerated storage conditions. J Agric Food Chem 2005;53(6):2103–10.
[8] Calpe-Berdiel L, Escolà-Gil JC, Blanco-Vaca F. New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. Atherosclerosis 2009;203(1):18–31.
[9] Reddy LH, Couvreur P. Squalene: a natural triterpene for use in disease management and therapy. Adv Drug Deliv Rev 2009;61(15):1412–26.

[10] Miettinen TA, Vanhanen H. Serum concentration and metabolism of cholesterol during rapeseed oil and squalene feeding. Am J Clin Nutr 1994; 59(2):356–63.
[11] Strandberg TE, Tilvis RS, Miettinen TA. Metabolic variables of cholesterol during squalene feeding in humans: comparison with cholestyramine treatment. J Lipid Res 1990;31(9):1637–43.
[12] Sotiroudis TG, Kyrtopoulos SA. Anticarcinogenic compounds of olive oil and related biomarkers. Eur J Nutr 2008;47(Suppl. 2):69–72.
[13] Kelly GS. Squalene and its potential clinical uses. Altern Med Rev 1999;4(1):29–36.
[14] Smith TJ. Squalene: potential chemopreventive agent. Expert Opin Investig Drugs 2000;9(8):1841–8.
[15] Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B, et al. Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol 2000;1(2):107–12.
[16] Warleta F, Campos M, Allouche Y, Sánchez-Quesada C, Ruiz-Mora J, Beltrán G, et al. Squalene protects against oxidative DNA damage in MCF10A human mammary epithelial cells but not in MCF7 and MDA-MB-231 human breast cancer cells. Food Chem Toxicol 2010;48(4):1092–100.
[17] Ostlund Jr RE, Racette SB, Stenson WF. Effects of trace components of dietary fat on cholesterol metabolism: phytosterols, oxysterols, and squalene. Nutr Rev 2002;60(11):349–59.
[18] Spanova M, Daum G. Squalene—biochemistry, molecular biology, process biotechnology, and applications. Eur J Lipid Sci Technol 2011; 113(11):1299–320.
[19] Kohno Y, Egawa Y, Itoh S, Nagaoka S, Takahashi M, Mukai K. Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in *n*-butanol. Biochim Biophys Acta 1995;1256(1):52–6.
[20] Kostyuk V, Potapovich A, Stancato A, de Luca C, Lulli D, Pastore S, et al. Photo-oxidation products of skin surface squalene mediate metabolic and inflammatory responses to solar UV in human keratinocytes. PLoS One 2012;7(8):e44472. http://dx.doi.org/10.1371/journal.pone.0044472.
[21] De Luca C, Valacchi G. Surface lipids as multifunctional mediators of skin responses to environmental stimuli. Mediators Inflamm 2010;2010:321–494. http://dx.doi.org/10.1155/2010/321494.
[22] Lindholm JS, Downing DT. Occurrence of squalene in skin surface lipids of the otter, the beaver and the kinkajou. Lipids 1980;15(12):1062–3.
[23] Ziboh VA, Miller CC, Cho Y. Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites. Am J Clin Nutr 2000;71(1 Suppl.):361S–6S.
[24] Richelle M, Sabatier M, Steiling H, Williamson G. Skin bioavailability of dietary vitamin E, carotenoids, polyphenols, vitamin C, zinc and selenium. Br J Nutr 2006;96(2):227–38.
[25] Boelsma E, Hendriks HF, Roza L. Nutritional skin care: health effects of micronutrients and fatty acids. Am J Clin Nutr 2001;73(5):853–64.
[26] Cho S, Choi CW, Lee DH, Won CH, Kim SM, Lee S, et al. High-dose squalene ingestion increases type I procollagen and decreases ultraviolet-induced DNA damage in human skin in vivo but is associated with transient adverse effects. Clin Exp Dermatol 2009;34(4):500–8.
[27] Fliesler SJ, Keller RK. Isoprenoid metabolism in the vertebrate retina. Int J Biochem Cell Biol 1997;29(6):877–94.
[28] Rao CV, Newmark HL, Reddy BS. Chemopreventive effect of squalene on colon cancer. Carcinogenesis 1998;19(2):287–90.
[29] Chan P, Tomlinson B, Lee CB, Lee YS. Effectiveness and safety of low-dose pravastatin and squalene, alone and in combination, in elderly patients with hypercholesterolemia. J Clin Pharmacol 1996;36(5):422–7.
[30] Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. Nutr Rev 2006;64 (Suppl. 1):S27–47.
[31] De Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 1999;99(6):779–85.
[32] López-Miranda J, Pérez Jiménez F, Ros E, De Caterina R, Badimón L, Covas MI, et al. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaén and Córdoba (Spain) 2008. Nutr Metab Cardiovasc Dis 2010;20(4):284–94.
[33] Lou-Bonafonte JM, Arnal C, Navarro MA, Osada J. Efficacy of bioactive compounds from extra virgin olive oil to modulate atherosclerosis development. Mol Nutr Food Res 2012;56(7):1043–57.
[34] Kritchevsky D, Moyer AW, Tesar WC. Squalene feeding in experimental atherosclerosis. Arch Biochem Biophys 1953;44(1):241.
[35] Guillen N, Acin S, Navarro MA, Perona JS, Arbonés-Mainar JM, Arnal C, et al. Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice. Atherosclerosis 2008;197(1):72–83.
[36] Bullon P, Quiles JL, Morillo JM, Rubini C, Goteri G, Granados-Principal S, et al. Gingival vascular damage in atherosclerotic rabbits: hydroxytyrosol and squalene benefits. Food Chem Toxicol 2009;47(9):2327–31.
[37] Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Ochoa-Herrera J, Perez-Lopez P, Pulido-Moran M, et al. Squalene ameliorates atherosclerotic lesions through the reduction of CD36 scavenger receptor expression in macrophages. Mol Nutr Food Res 2012;56(5):733–40.
[38] Sabeena Farvin KH, Anandan R, Kumar SHS, Shiny KS, Sankar TV, Thankappan TK. Effect of squalene on tissue defense system in isoproterenol-induced myocardial infarction in rats. Pharmacol Res 2004;50(3):231–6.
[39] Motawi TMK, Sadik NAH, Refaat A. Cytoprotective effects of DL-alpha-lipoic acid or squalene on cyclophosphamide-induced oxidative injury: an experimental study on rat myocardium, testicles and urinary bladder. Food Chem Toxicol 2010;48(8–9):2326–36.
[40] Psaltopoulou T, Kosti RI, Haidopoulos D, Dimopoulos M, Panagiotakos DB. Olive oil intake is inversely related to cancer prevalence: a systematic review and a meta-analysis of 13,800 patients and 23,340 controls in 19 observational studies. Lipids Health Dis 2011;10:127–33.
[41] Newmark HL. Squalene, olive oil, and cancer risk: a review and hypothesis. Cancer Epidemiol Biomarkers Prev 1997;6(12):1101–3.
[42] Murakoshi M, Nishino H, Tokuda H, Iwashima A, Okuzumi J, Kitano H, et al. Inhibition by squalene of the tumor-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate in mouse-skin carcinogenesis. Int J Cancer 1992;52(6):950–2.
[43] Ohkuma T, Otagiri K, Tanaka S, Ikekawa T. Intensification of host's immunity by squalene in sarcoma 180 bearing ICR mice. J Pharmacobiodyn 1983;6(2):148–51.

[44] Smith TJ, Yang GY, Seril DN, Liao J, Kim S. Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis by dietary olive oil and squalene. Carcinogenesis 1998;19(4):703–6.
[45] Visioli F, Grande S, Bogani P, Galli C. The role of antioxidants in the Mediterranean diets: focus on cancer. Eur J Cancer Prev 2004;13(4):337–43.
[46] Tilvis R, Kovanen PT, Miettinen TA. Metabolism of squalene in human fat cells. Demonstration of a two-pool system. J Biol Chem 1982;257 (17):10300–5.
[47] Escrich E, Ramírez-Tortosa MC, Sánchez-Rovira P, Colomer R, Solanas M, Gaforio JJ. Olive oil in cancer prevention and progression. Nutr Rev 2006;64(10 Suppl. 1):S40–52.
[48] Das B, Antoon R, Tsuchida R, Lofti S, Morozova O, Farhat W, et al. Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity in vivo without protecting tumor growth. Neoplasia 2008;10(10):1105–19.
[49] Das B, Yeger H, Baruchel H, Freedman MH, Koren G, Baruchel S. In vitro cytoprotective activity of squalene on a bone marrow versus neuroblastoma model of cisplatin-induced toxicity: implications in cancer chemotherapy. Eur J Cancer 2003;39(17):2556–65.
[50] Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 2004;44:239–67.
[51] Owen RW, Haubner R, Wurtele G, Hull E, Spiegelhalder B, Bartsch H. Olives and olive oil in cancer prevention. Eur J Cancer Prev 2004; 13(4):319–26.

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El Sector de Elaboración de ACEITE DE OLIVA:

Un estudio multidisciplinar

Prólogo de Fernando Burgaz Moreno

Ministerio de Agricultura, Alimentación y Medio Ambiente

Coordina: Juan Vilar Hernández

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DECLARACIONES AUTORIZADAS DE PROPIEDADES SALUDABLES EN EL ACEITE DE OLIVA

Autores

José Juan Gaforio Martínez
Fernando Warleta Arias
Cristina Sánchez-Quesada
Alicia López-Biedma

1. INTRODUCCIÓN

El conocimiento que el consumidor general posee sobre el aceite de oliva es limitado, incluso, lo podríamos catalogar de confuso. Así los corroboran distintos estudios realizados sobre este tema. Son esclarecedores los resultados obtenidos en un estudio recientemente publicado (abril de 2012) y realizado por la Consejería de Agricultura y Pesca de la Junta de Andalucía. En él se constata que, el 46% de los encuestados no identifica bien los tipos de aceites de oliva; el 12% desconoce que el aceite de oliva virgen extra es el que tiene más calidad; el 30% manifiesta que se guía por el precio para adquirir el aceite; y el 51% asegura que se guía por la categoría de este, aun constatando que un porcentaje muy alto de consumidores es incapaz de diferenciar las distintas categorías y características de los aceites de oliva.

Por otra parte, el sector productor muestra, desde hace tiempo, su desazón por los bajos precios en origen y por la insuficiente retribución del aceite de calidad (aceite de oliva virgen extra), cuestionando directamente la viabilidad futura de este cultivo.

La revalorización del sector oleícola pasa por diversas y complejas cuestiones pero, entiendo que, el eje sobre el que debe girar su futuro es el "Consumidor". Es únicamente éste el que puede hacerlo rentable si, en primer lugar valora y, finalmente, prioriza la compra del aceite de oliva por delante de otras opciones. No debemos olvidar que "*Compramos lo que conocemos y valoramos*". Un objetivo prioritario debe ser pues, informar y dar a conocer las características del producto al consumidor para que éste, posea los conocimientos necesarios para poder hacer una compra inteligente.

Las nuevas generaciones de consumidores se caracterizan por tener a su disposición, gracias a internet, una gran cantidad de información, de tener una mayor capacidad de decisión y, de compartir activamente sus opiniones y gustos a través de las redes sociales. La difusión de información y noticias por esta vía es portentosa gracias a su inmediatez y capacidad para llegar a un gran número de personas. No obstante, esto es un arma de doble filo pues, no todo lo que se difunde por esta vía tiene por qué ser cierto y estar sustentado en datos contrastados. En bastantes ocasiones, nos encontramos con reseñas de dudosa verosimilitud cuando no directamente falaces. Estos son factores relativamente nuevos que hay que tener siempre presentes.

Aunque resultan evidentes, quiero comenzar este apartado señalando que: (1) no es obligatorio consumir aceite de oliva; (2) hay otras grasas comestibles, que son

consumidas mayoritariamente, concretamente, el consumo de aceite de oliva representa menos del 3% de las grasas vegetales consumidas en el mundo; (3) como norma, estas grasas alternativas son más económicas que el aceite de oliva; (4) para muchas sociedades, el aceite de oliva virgen es una grasa ajena a su cultura culinaria y les resulta chocante su sabor, e incluso, lo describen como no agradable a su paladar.

Estos datos ponen de manifiesto que el consumidor dispone de alternativas al aceite de oliva que, además, son más económicas, y esto siempre es un dato muy a considerar, sobre todo, en tiempos de crisis económica. Puesto que en el mercado hay alternativas más baratas, si los consumidores no aprecian otras cualidades que las diferencien, la opción de compra será obvia. Es pues imprescindible proporcionar argumentos a los consumidores para que su opción sea el aceite de oliva.

El acto de compra se sustenta en satisfacer las necesidades del individuo. Diferentes estudios de mercado ponen de manifiesto que, entre los factores de discriminación más importantes que motivan a los consumidores en la compra de productos alimentarios, se encuentra la salud. Hay un movimiento creciente en las nuevas sociedades que fomenta y valora la salud y el aspecto físico y, todo el mundo es consciente que en ambos aspectos, la alimentación (junto con el ejercicio físico) juega un papel decisivo. Las evidencias científicas corroboran plenamente esta asociación. Como consecuencia de ello, una parte significativa de la población está cada día más motivada a consumir una dieta equilibrada y sana que redunde positivamente en su bienestar. Muestra de ello es el auge de los denominados "alimentos funcionales". No existe una definición oficial sobre ellos pero, la más aceptada es la que dice que, un alimento se puede considerar funcional si se demuestra satisfactoriamente que, además de sus efectos nutritivos, afecta beneficiosamente a una o más funciones del organismo de modo que mejore el estado de salud o bienestar o reduce el riesgo de enfermedad.

En este contexto, las propiedades saludables del aceite de oliva son un baluarte inapreciable para su comercialización, al consolidar su diferenciación de otras grasas comestibles.

Aprovechando la tendencia social anteriormente anotada, la industria agroalimentaria está utilizando intensivamente en la publicidad las propiedades saludables

como mecanismo para diferenciar y comercializar sus productos. No obstante, surgen dudas sobre la veracidad de estas propiedades en algunos casos y, los consumidores se preguntan si es cierto todo lo que se publicita. Ante tales recelos que conducen a la desprotección del consumidor, había que arbitrar disposiciones legales que protejan a estos frente a la publicidad que esgrime propiedades saludables con insuficiente o nulo fundamento científico, al mismo tiempo que, prime aquellos alimentos que posean dichas propiedades.

El Reglamento (CE) nº 1924/2006 del Parlamento Europeo y del Consejo de 20 de diciembre 2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos armoniza las disposiciones legales, reglamentarias o administrativas de los Estados miembros relativas a las declaraciones nutricionales y de propiedades saludables con el fin de garantizar un funcionamiento eficaz del mercado interior a la vez que proporciona un elevado nivel de protección de los consumidores. Este Reglamento se aplica a las declaraciones nutricionales y de propiedades saludables efectuadas en las comunicaciones comerciales, ya sea en el etiquetado, la presentación o la publicidad de los alimentos que se suministren como tales al consumidor final. Una de las finalidades de este Reglamento es garantizar que las declaraciones de propiedades saludables sean veraces, claras, fiables y útiles para el consumidor, y para ello, se fija que el fundamento científico es el principal aspecto a considerar para el uso tanto de las declaraciones nutricionales como de propiedades saludables. Para la fundamentación científica, se toma en consideración la totalidad de los datos científicos disponibles y la ponderación de las pruebas. Este Reglamento, en su artículo 10, apartado 1, dispone que están prohibidas las declaraciones de propiedades saludables de los alimentos a no ser que las autorice la Comisión de conformidad con este Reglamento y las incluya en una lista de declaraciones autorizadas.

El Diario Oficial de la Unión Europea, publicó el 5 de mayo de 2012 el Reglamento (UE) nº 432/2012 de la Comisión de 16 de mayo de 2012 por la que se establece una lista de Declaraciones autorizadas de propiedades saludables de los alimentos, adoptada por la Comisión previa consulta a la Autoridad Europea de Seguridad Alimentaria, tal y como estipula el artículo 13, apartado 3, del Reglamento (CE) nº 1924/2006. El Reglamento será aplicable a partir del 14 de diciembre de 2012.

Tres son los nutrientes presentes en esta lista integrantes del aceite de oliva y por consiguiente, pueden ser utilizadas sus declaraciones de propiedades saludables en su comercialización. Los nutrientes, declaraciones autorizadas y condiciones de uso de estas, son:

2. ÁCIDO OLEICO

- Declaración autorizada: la sustitución de grasas saturadas por grasas insaturadas en la dieta contribuye a mantener niveles normales de colesterol sanguíneo. El ácido oleico es una grasa insaturada.
- Condiciones de uso de la declaración: esta declaración solo puede utilizarse respecto a alimentos con alto contenido de ácidos grasos insaturados, de acuerdo con la declaración de ALTO CONTENIDO DE GRASAS INSATURADAS que figura en el anexo del Reglamento (CE) nº 1924/2006.

3. VITAMINA E

- Declaración autorizada: la vitamina E contribuye a la protección de las células frente al daño oxidativo.
- Condiciones de uso de la declaración: esta declaración solo puede utilizarse respecto a alimentos que son, como mínimo, fuente de vitamina E de acuerdo con la declaración FUENTE DE (NOMBRE DE LAS VITAMINAS) Y/O (NOMBRE DE LOS MINERALES) que figura en el anexo del Reglamento (CE) nº 1924/2006.

4. POLIFENOLES DEL ACEITE DE OLIVA

- Declaración autorizada: los polifenoles del aceite de oliva contribuyen a la protección de los lípidos de la sangre frente al daño oxidativo.
- Condiciones de uso de la declaración: Esta declaración solo puede utilizarse respecto a aceite de oliva que contenga un mínimo de 5 mg de hidroxitirosol y sus derivados (por ejemplo, un complejo de oleuropeína o tirosol) por 20 g de aceite de oliva. Para que un producto pueda llevar esta declaración, se informará al consumidor de que el efecto beneficioso se obtiene con una ingesta diaria de 20 g de aceite de oliva.

Estas declaraciones demuestran que hay base científica sólida que avala los efectos beneficiosos que el consumo de aceite de oliva tiene sobre nuestra salud. Esto posibilita su utilización en el etiquetado y la publicidad del aceite de oliva por lo que será una herramienta de primer orden que hay que saber aprovechar en beneficio del sector. El aval que esto supone será muy beneficioso para los consumidores, al ofrecer información veraz y protegerlos de la publicidad engañosa.

La última de las declaraciones autorizadas (polifenoles del aceite de oliva), abre una puerta especialmente interesante pues, se especifica la cantidad mínima de estos que debe tener el aceite de oliva para que pueda ser utilizada. Significa ello que, no todos los aceites de oliva vírgenes podrán usar esta declaración al variar de forma importante la composición en los distintos aceites de oliva vírgenes dependiendo fundamentalmente, de la variedad de aceituna de la que proceden.

Hay que resaltar que la Comisión establece y mantiene el registro comunitario de declaraciones nutricionales y de propiedades saludables relativas a alimentos. Aclarar que, no es una lista cerrada, por el contrario, podrán incluirse nuevas declaraciones, de la misma forma que se podrán modificar, suspender o revocar las autorizadas. Quiere esto decir que podrá autorizarse en el futuro nuevas declaraciones relacionadas con el aceite de oliva, pero para ello, será fundamental persistir y consolidar las investigaciones que aportarán las evidencias científicas necesarias para conseguir este fin.

5. COLOFÓN

El comentario final lo dedicaremos a las perspectivas de futuro. Como hemos apuntado anteriormente, este sector no pasa por su mejor momento. Estamos convencidos que tiene futuro, pero con solo creerlo, nada podemos arreglar. Hay que ser proactivos, centrando nuestros esfuerzos en las cosas con respecto a las cuales podemos hacer algo, asumiendo el papel que nos corresponde como líderes mundiales en producción. Desde hace demasiado tiempo, hay una tendencia a la queja y una actitud lastimera. Con eso, se da la sensación que son otros los responsables de nuestros problemas y que, también tienen que ser otros los que nos saquen de este atolladero. Culpar a los demás y al entorno de nuestros problemas y dificultades, nos encadena a esos mismos problemas. Hasta tanto no seamos conscientes que tenemos que ser nosotros los artífices y protagonistas de este cambio, nada podremos conseguir. Debemos aceptar y asumir la responsabilidad de nuestras circunstancias y, ser lo suficientemente valientes como para tomar las iniciativas que sean necesarias para afrontar creativamente la solución de nuestros problemas.

Como en cualquier otro caso en el que queramos abordar la resolución de un problema, tendremos que seguir una serie de pasos, a saber: definir y analizar el problema; definir objetivo; evaluar alternativas de solución; elegir alternativa; decidir plan de acción; aplicar decisión. Cada uno, con sus particularidades, deberá hacer su propio planteamiento. Hablaremos aquí brevemente de algunas ideas generales.

El punto de partida es que somos líderes mundiales en producción; las exportaciones en los dos últimos años han alcanzado cifras inimaginables con anterioridad; sin embargo, el precio del aceite de oliva es muy bajo poniendo en peligro su rentabilidad. El objetivo general que nos tenemos que plantear es hacerlo un cultivo competitivo y sostenible medioambientalmente.

Bajo nuestro punto de vista, algunas de las bases para alcanzar el objetivo, son:

- Profesionalización. Es habitual olvidarse que la cooperativa de muchos de nuestros pueblos es la empresa con mayor facturación de todas las que posee. Sin embargo, habitualmente no se gestiona de forma profesional, como cualquier empresa de otros sectores. Este es un paso transcendental para dar entrada en su organigrama a personas con la preparación idónea para hacerla competitiva introduciendo una mentalidad empresarial actual.

- Respeto al medio-ambiente. Tiene que ser un cultivo sostenible donde el cuidado y respeto del medio ambiente tiene que ser un objetivo innegociable. Una prioridad tiene que ser la reducción, o incluso eliminación (cuando ello sea posible), de plaguicidas, herbicidas y fertilizantes; priorizar el uso de energías renovables y la optimización de uso del agua; incentivar las cubiertas vegetales que impiden la erosión y favorecen la biodiversidad; proteger e impulsar la presencia de vegetación natural en lindes, setos, árboles aislados, bordes de montes, riberas de arroyos, etc. Esto es de tal importancia que incluso vendrá recogido en la nueva PAC y donde se estipula que una parte importante de los pagos directos serán en concepto de reverdecimiento.
- Seguridad alimentaria. Hay que apostar por la elaboración de aceites de oliva de calidad. La lucha frente al fraude tiene que ser implacable así como el control de residuos.
- Investigación e innovación. Ya hemos comentado anteriormente que las declaraciones de propiedades saludables se sustentan en las evidencias científicas, por lo que es fundamental persistir en la investigación en este terreno que, por añadidura, posibilita la diferenciación del aceite de oliva del resto de grasas comestibles. Por otra parte, el motor del cambio tiene que ser la innovación afrontada desde distintas perspectivas: de producto, en la orientación al cliente, en las operaciones y organización y, por último, en el marketing.

Hay por delante un futuro prometedor pero en el que hay que trabajar duro para afrontar los retos. Este cultivo es crucial para España pues, históricamente, ha sido la locomotora económica de muchos pueblos de nuestro País. Nuestra situación de líderes mundiales en producción, nos obliga a asumir un papel estelar para impulsar su competitividad y sostenibilidad en las próximas décadas.

Anexo IV

Aportaciones a congresos

1) "Antitumor effect of pinoresinol on human breast cancer cells". 5th Euro-Global Summit and Expo on Food & Beverages. Alicante, Comunidad Valenciana, España. 16/06/2015 a 18/06/2015. OMICS international. López-Biedma A; Sánchez Quesada C; Gaforio JJ.

2) "Efecto antitumoral del Pinoresinol en células tumorales de mama humanas". XVII simposium cientifico-técnico. Jaén, Andalucía, España. 06/05/2015 a 08/05/2015. Fundación para la promoción y el desarrollo del olivar y del aceite de oliva. López-Biedma A; Sánchez Quesada C; Gaforio JJ.

3) "La localización de un grupo metilo causa diferente efecto antitumoral entre dos compuestos del aceite de oliva". XVII simposium cientifico-técnico. Jaén, Andalucía, España. 06/05/2015 a 08/05/2015. Fundación para la promoción y el desarrollo del olivar y del aceite de oliva.Sánchez-Quesada C; López-Biedma A; Gaforio JJ.

4) "Disminución de la capacidad de invasión, implicada en el proceso metastásico de la línea tumoral mamaria MDA-MB-231 tras tratamiento con Tirosol". XVI simposio científico-técnico del aceite de oliva. Jaén, Andalucía, España. 08/05/2013 a 10/05/2013. Fundación para la promoción y el desarrollo del olivar y del aceite de oliva. López-Biedma A; Sánchez Quesada C; Warleta F; Gaforio JJ.

5) "Acción del escualeno, componente minoritario del aceite de oliva virgen, sobre células tumorales de mama". 31 Reunión nacional de la sección de ginecología oncológica y patología mamaria de S.E.G.O. Sevilla, Andalucía, España. 26/10/2012 a 28/10/2012. Sociedad española de ginecología y obstreticia. García Carriazo M; Warleta F; Sánchez Quesada C; López Biedma A; Gaforio JJ.

6) "Determinación de la capacidad antioxidante y antitumoral de la oleuropeína y principales lignanos presentes en el aceite de oliva virgen". Estudio sobre células tumorales de mama humanas. I Congreso Científico de Investigadores en Formación en Agroalimentación. Córdoba, Andalucía, España. 08/05/2012 a 09/05/2012. Universidad de Córdoba. López-Biedma A.

Anexo V

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