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Effect of a Purified Extract of Olive Mill Waste Waters on Endothelial Cell Proliferation, Apoptosis, Migration and Capillary-Like Structure *in vitro* and *in vivo*

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Abstract

Olive oil, a major feature of the Mediterranean diet, is an important source of phenolic compounds. Poliphenols are associated with inhibition of several pathological processes, including cancer. Soluble phenolic compounds are contained in the aqueous part of olive and are discarded during oil production in the 'olive mill wastewaters'. Here we investigated the properties of a purified extract of olive mill wastewaters, named A009, as potential anti-angiogenic compound. While the strong anti-oxidant activity of olive derived phenolic compounds has been well characterized, little is known about their anti-angiogenic properties. We investigated effects of A009 on endothelial cell morphogenesis, proliferation, migration and apoptosis, comparing the results obtained with the activity of a well-characterized olive oil phenol, Hydroxytyrosol (HT). Further, we tested the effects of A009 and HT in an *in vivo* angiogenesis assay. We found that A009 exerted strong anti-angiogenic effects both *in vitro* and *in vivo*, and that the complex natural purified extract has stronger anti-angiogenic potential when compared to the same concentrations of HT in most of the assays performed. These data demonstrate that a novel purified, polyphenol enriched extract with anti-angiogenic, and angiopreventive potential can be obtained from olive oil mill waste material, recovering useful products from an agricultural waste.

Keywords: Angiogenesis; Olive mill wastewaters; Olive oil; Hydroxytyrosol; Angioprevention; Phenols

Introduction

Olive oil represents a common component of the 'Mediterranean Diet' (MD), which has been extensively reported, along with healthy lifestyle habits, to be associated with decreased chronic conditions [1], including diabetes [2], cardiovascular diseases (CV) [3] and cancer [4]. Several epidemiological studies have shown that the incidence of several tumors is relatively low in areas where the Mediterranean diet dominates as compared to other European or North America countries [5-8].

Olive oil contains a phenolic fraction that has been reported to exert chemopreventive effects [9]. Chemoprevention [10] is the concept of preventing cancer by the administration of bioactive molecules to block, revert or delay the carcinogenic process [11]. These mechanisms include angiogenesis inhibition [12] and induction of apoptosis [13]. Among the phenols of olive oil, the most studied is hydroxytyrosol (HT) [14]. The biological properties of HT, including anti-oxidant [15], anti-apoptotic [16], anti-tumor [17] and anti-inflammatory activities [18], have been recently demonstrated by several *in vitro* and *in vivo* studies and have also been recognized by the European Food Safety Authority (EFSA).

Most of the studies concerning the benefits associated with olive oil consumption have been focused on the final product. Several processes are required for the production of high quality olive oil, which are in turn associated with waste management at each production stage. Among the waste products derived from oil production, an increased interest has been addressed to the olive-derived aqueous liquor, termed olive mill wastewater (OMWW). This waste product is highly enriched in phenol content [19], and increasing attention has been addressed to test OMWW as a source of promising bioactive compounds. The crucial points regarding the potential use of OMWW as nutraceutical, are associated with diverse factors, including the type and maturation state of olives, agricultural region, climatic conditions, related agro-technology/processing methods and oil extraction procedures [20]. These features are crucial in determining the OMWW phenolic fraction composition, which has been observed to range from 0.5 to 24 g/L [21].

While the anti-tumor effects of the phenolic compounds are largely studied [22], little is known about their anti-angiogenic and angiopreventive potential referred as the ability to target angiogenesis in cancer prevention [23]. Angiogenesis, the formation of new blood vessels from preexisting vasculature, is essential for many physiological processes, such as embryogenesis, and also plays a critical role in many pathological conditions, including cancer. This process is an essential event for the tumor growth and a crucial hallmark of cancer progression and metastasis [24]. Several diet derived compounds and their synthetic derivatives have been reported to exert anti-oxidant, anti-tumor [25], anti-angiogenic and angiopreventive activities [26] in a wide array of hematologic and solid malignancies.

Therefore, the aim of the present study was to evaluate the anti-angiogenic and angiopreventive potential of an highly phenol-rich olive mill wastewater purified extract, termed A009, on endothelial cell

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proliferation, induction of apoptosis, migration and network formation activities *in vitro* and its ability to interfere with angiogenesis *in vivo*. Furthermore, we demonstrate that the A009 phenol-rich extract exerts a more potent angiopreventive/anti-angiogenic activity as compared to purified HT alone. Our results underline the possibility to derive promising bioactive compounds from an agricultural waste product, providing a valid dietary supplement while containing waste product.

Materials and Methods

Reagents

OMWW were kindly provided by Agriturismo La Vialla (Castiglion Fibocchi (Arezzo), Italy). A009 a phenol rich purified extract was obtained from Massimo and Daniele Pizzichini according to Patent formulation (Patent 8815815). All experiments were performed using 2 different A009 batches. This is an important control to insure activity is not altered in different batches due to potential variations in chemical composition and compounds present in the extract that might be influenced by certain conditions including seasonal weather variations and the times between olive oil extraction and extract preparation. Hydroxytyrosol (HT), synthetic in origin whose purity is ≥ 98%, was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Ethanol (EtOH, the vehicle for HT) was purchased from Sigma-Aldrich (Milan, Italy).

Preparation of A009 and phenolic quantification

Olive extract was obtained from OMWW using two sequential cross-flow filtration process. First, a ceramic Microfiltration (MF) was performed using 2 tubular membranes in alumina oxide with a 300 KDa cut-off 3 (TAMI membranes, Nyons, France) and a filtering surface of 0.35 m^2. This removes solid particles, residual plant particles and cells that were discarded. The MF permeate was further concentrated by reverse osmosis (RO) in a Polyamide spiral wound module (Microdyn Nadir, Wiesbaden, Germany) with a filtering surface of 7 m^2. The RO permeate was essentially ultrapure water and was discarded. Finally, the RO concentrate, obtained reaching a volume concentration ratio (VCR) of 3.6, constituted the olive extract (here termed A009). Quantification of the phenolic presence in A009 was performed using a high-performance liquid chromatography (HPLC) analysis. Quantification revealed that the major phenolic component of A009 was hydroxytyrosol in both the batches (2.7 and 5.72 gr/L), along with several other phenolic compounds (Table S1).

Phenolic compound (g/L)	A009 batch 1	A009 batch 2
Hydroxytyrosol glucoside	ND	1,69
Hydroxytyrosol	2.7	5.72
Tyrosol	0.2	ND
Chlorogenic acid	0.12	0.1
B-hydroxyverbascoside isomer 1	0.35	0.14
B-hydroxyverbascoside isomer 2	0.32	0.17
Verbascoside	0.84	1.32
caffeyol ester of secologanoside	ND	0,2
Decarboxymethyloleuropeinaglycon	1.99	0.28
Oleouropein aglycon	ND	0.22
6'-p-coumaroyl secologanoside	ND	0.4
Rutin	0.11	ND
luteolin-7-o-glucoside	0.22	ND

Table S1: Phenolic quantification in OMWW extract. Quantification of the phenolic presence in A009 was performed using an highperformance liquid chromatography (HPLC) analysis. Results are expressed as g/L. N.D. Not detected.

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Promo Cell (Heidelberg, Germany) and grown on 1% gelatin-coated tissue culture plates in Medium 199 (Sigma Aldrich Milan, Italy), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Euroclone, Milan, Italy), 1% glutamine (Euroclone, Milan, Italy), fibroblast growth factors (1 µg/100 ml acid-fibroblast growth factor plus 1µg/ 100ml basic-fibroblast growth factor, PeproTech London UK), epidermal growth factor (1 µg/100 ml PeproTech London UK), heparin (10 mg/100 ml, Sigma Aldrich Milan, Italy) and hydrocortisone (0.1 mg/100 ml Sigma Aldrich Milan, Italy) at 37°C in 5% CO_2. Cells were used between the second and sixth passage *in vitro*.

In vitro cell proliferation

HUVEC proliferation was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich, Milan, Italy) assay. Cells (10^3/well) were seeded into 96-multiwell plates in complete medium. After cell adhesion, fresh complete medium with decreasing dilutions of treatments was added as follows: A009 (1:10000-1:100), HT (1:10000-1:100) and EtOH (1:10000-1:100). Cells were treated for 24, 48, 72 and 96 hours and the relative absorbance was measured at 540 nm by a FLUOstar spectrophotometer (FLUOstar Omega BMG LABTECH, Ortenberg, Germany).

Apoptosis assay

HUVECs (1.5 × 10^5 cells/well) were plated in 6-well plates and incubated with decreasing dilutions (1:2500-1:250) of A009, HT or EtOH for 24h and 48h in 10% FBS-supplemented complete medium. Cells were recovered, washed twice with PBS and transferred to test tubes. Cells were pelleted by centrifugation, resuspended in Annexin V-binding buffer (BD Biosciences, Milan, Italy) and stained with Fluorescein isothiocyanate (FITC)-conjugated Annexin V and 7-amino-actinomycin D (7-AAD) (BD Biosciences Milan, Italy) for 15 min at 4°C in the dark. Cells were then washed in PBS, supernatants discarded and resuspended in 400 µL of PBS. Analysis was performed by flow cytometry using a FACSCanto (BD Biosciences, Milan, Italy) on FSC/SSC viable gated cells, excluding cell debris, with excitation set at 488 nm and emission at 518 nm (FITC detector) and 620 nm (PE detector). Data were analyzed using FACSDiva Software 6.1.2. The experiment was performed three times and each condition was in duplicate.

Induction of reactive oxygen species (ROS)

The ability of A009 to inhibit intracellular ROS generation on treated or pre-treated endothelial cells was determined using 2,7-dichlorofluorescin diacetate (DCFH-DA; Sigma Aldrich, Milan, Italy) assay coupled with flow cytometry based on the previously described protocol [27]. The assay was performed by 2',7'-dichlorofluorescin diacetate staining (H_2DCF-DA) in order to detect and localize intracellular sources of ROS. Non-ionized H_2DCF-DA is membrane permeant and therefore is able to diffuse readily into cells, once within the cell, the acetate groups are hydrolysed by intracellular esterase activity forming 2',7'- dichlorodihydrofluorescein (H_2DCF) which is polar and thus trapped within the cell. H_2DCF fluoresces when it is oxidized by H_2O_2 or lipid peroxides to yield 2',7'-dichlorofluorescein (DCF). The level of DCF produced within

the cells is related linearly to that of peroxides present and thus its fluorescent emission provides a measure of the peroxide levels.

A009 pre-treatment of endothelial cells was performed by transferring 1.5×10^5 HUVECs to test tubes and incubating them with decreasing dilutions (1:500-1:250) of A009, HT or EtOH in complete medium for 30 minutes at 37°C, 5% CO_2. Cells were then resuspended in PBS and incubated with H_2DCF-DA (10 μM) and H_2O_2 (250 μM) for 45 minutes at 37°C, 5% CO_2. The fluorescence intensity (525 nm) was detected using a FACSCanto (BD Biosciences, Milan, Italy) flow cytometer.

For H_2O_2 pre-treatment, 1.5×10^5 HUVECs were transferred to test tubes in complete medium and incubated with H_2O_2 (250 μM) for 15 minutes at 37°C and 5% CO_2. Cells were then resuspended in PBS and incubated with H_2DCF-DA (10 μM) and decreasing dilutions (1:500-1:250) of A009, HT or EtOH for 45 minutes at 37°C and 5% CO_2. The fluorescence (525 nm) was measured using a FACSCanto (BD Biosciences, Milan, Italy) flow cytometer.

Migration and invasion assays

Chemoinvasion and chemotaxis assays were performed using modified Boyden chambers, as previously described [28,29]. HUVECs (5×10^4) were pre-treated with decreasing dilutions (1:500-1:250) of A009, HT or EtOH as a vehicle for 24 h, then washed in PBS, resuspended in serum-free medium and placed in the upper compartment of the chamber. M199 medium supplemented with 10% FBS as attractant factor was added in the lower chamber compartment. 8 μm pore-size polycarbonate filters (Whatman, GE Healthcare Europe GmbH, Milan, Italy) were pre-coated with collagen IV (50 μg/mL, Sigma Aldrich, Milan, Italy) for the chemotaxis assay or matrigel (1 mg/mL, BD Biosciences, Milan, Italy) for the chemoinvasion assay. Cells were incubated for 6 (chemotaxis) or 24 (chemoinvasion) hours at 37°C, filters were then recovered, cells on the upper surface mechanically removed with a cotton swab and cells migrated or invaded toward the lower filter surface fixed with absolute ethanol and stained with DAPI (Vectashield, Vector Laboratories, Orton Southgate, Peterborough, United Kingdom). Cells were counted in a double-blind manner in five consecutive fields with a fluorescent microscope (Nikon Eclipse Ni). All experiments were performed three times in duplicate.

Morphogenesis assay on matrigel

The ability of A009, HT or EtOH to interfere with the capillary-like network formation was assessed by the morphogenesis assay *in vitro* [30]. A 24-well plate, pre-chilled at -20°C, was carefully filled with 300 μL per well of liquid matrigel (BD Biosciences, Milan, Italy) at 4°C with a pre-chilled pipette, avoiding bubbles and polymerized for 1h at 37°C. HUVECs (5×10^4 cells/well) were suspended in 1 mL of complete medium supplemented with 10% FBS in the presence of decreasing dilution (1:500-1:250) of A009, HT or EtOH and carefully layered on the top of the polymerized matrigel. Effects on the growth and morphogenesis of HUVECs were recorded after 6 h incubation with an inverted microscope (Nikon Eclipse TS100). The number of segments and their length were quantified using ImageJ software and the "Angiogenesis Analyzer" tool.

In vivo matrigel sponge assay

The ability of A009 to inhibit angiogenesis *in vivo* was assessed by the matrigel sponge assay, as already described [31]. A009 or HT (dilution 1:500 and 1:250) were added to unpolymerized liquid matrigel, containing an heparin (25 U/mL), VEGF (100 ng/μL) and TNF-α (1.2 ng/ μL) mixture (VTH), and slowly subcutaneously injected into the flanks of 6- to 8-week-old C57/BL6 male mice (Charles River Laboratories, Calco (Lecco), Italy) in groups of 4 animals for each treatment. The animal care and experimental protocols were performed according to the Italian Approved Animal Welfare Assurance. Following four days after injection, mice were sacrificed and the pellets were removed, weighted, and cryostored using OCT for histological examination, or minced and diluted in PBS for hemoglobin content measurement. Hemoglobin content was determined using 200 μL of the supernatant derived from minced pellets, centrifuged for 12 minutes 13000 g at 4°C and mixed with 800 μL of Drabkin reagent (Sigma Aldrich, Milan, Italy). The final hemoglobin concentration was calculated from a standard calibration curve, by spectrophotometric analysis at 540 nm (FLUOstar Omega BMG LABTECH, Ortenberg, Germany). The hemoglobin concentration was calculated as: (Abs 540 nm/pellet weight mg) × 100.

Immunohystochemical analysis

Sections of 5 μm thickness from OCT cryostored samples were stained with hematoxylin and eosin for histological examination following standard procedures. The sections were photographed on an inverted microscope (Nikon Eclipse TS100).

Statistical analysis

Results are showed as mean ± SD. The significance of differences was evaluated with a two-tailed t-test using Prism software (GraphPad Software for Science, Inc., San Diego, CA, USA).

Results

A009 inhibits HUVECs proliferation

A009 was tested for its ability to interfere with endothelial cell proliferation, and compared with HT alone at a similar concentration (2.7 g/L) as that contained for A009 (Table S1). A009 was able to inhibit HUVEC proliferation starting from 24 hours at 1:1000 dilution, while higher concentrations of A009 completely arrested cell proliferation (Figure 1A). When compared to a similar dilution of HT alone, HT showed a lesser effect on cell proliferation, suggesting that the presence of heterogeneous complex of phenolics in the A009 extract enhances anti-proliferative activity. Finally, EtOH, the vehicle for HT, showed no effect on HUVEC proliferation (data not shown). The IC50 of A009 was determined using the concentration of HT presented in the pool as shown in Figure 1B.

A009 induces apoptosis on endothelial cells

We evaluated whether the cytostatic effect of A009 was associated with the induction of apoptosis using a flow cytometry based apoptosis assay. Following 24 hours of treatment with A009 (1:500 and 1:250) 30% and 50%, respectively, of HUVECs had entered into apoptosis, as detected by AnnexinV and 7-AAD positivity (Figure 2A). In contrast, HUVECs exposed to HT alone, at the same dilution ranges (1:2500-1:250) as those for A009, showed only low apoptotic rates (15% of $AnnexinV^+7\text{-}AAD^+$ cells at 1:250 dilution). After 48 hours of treatment, HUVECs showed increased apoptosis with A009 as compared to HT alone (Figure 2B). Cells treated with vehicle showed very low apoptosis.

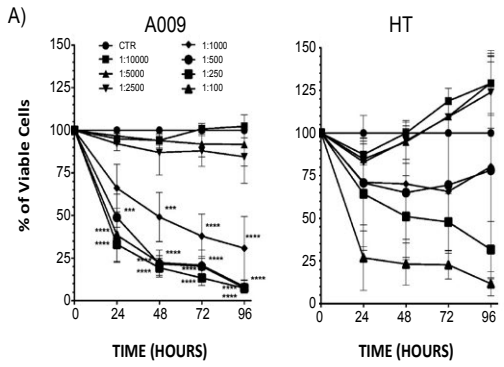
A009 inhibits ROS production before and after H_2O_2 treatment

Oxidative stress occurs when disequilibrium between reactive

oxygen species (ROS) production and host antioxidant capacity is present. Reactive Oxygen Species are induced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxide-reductase enzymes and metal catalyzed oxidations, and are associated with several inflammatory conditions. Given the antioxidant compounds present in A009, we evaluated the scavenger ability of A009 on HUVECs following pre-treatment with H_2O_2 or A009, as compared to HT alone. A009 showed strong ROS scavenger effects, significantly decreasing the H_2DCF-DA fluorescence signal, both in pre-treatment (Figure 3A, $p<0.001$ for 1:500 and 1:250 dilution), and post-treatment (Figure 3B $p<0.001$; for 1:500 and 1:250 dilutions). HT alone exerted little ROS scavenger activities, supporting the hypothesis of an enhanced anti-oxidant effect in A009.

A009 inhibits endothelial morphogenesis *in vitro*

When cultured on matrix layer supplemented with pro-angiogenic factors, HUVECs are able to organize into capillary-like networks. We observed that A009 was able to interfere with HUVECs morphogenesis in a dose-dependent manner to a similar extent as HT alone (Figure 4A). The quantification of the number of meshes (Figure 4B) indicated a statistically significant effect of both A009 (1:250 $p<0.05$) and HT (1:250 $p<0.05$).



B)

	24h	48h	72h	96h
A009	142.90 µM	74.50 µM	66.92 µM	58.15 µM
HT	201.92 µM	134.84 µM	128.11 µM	116.35 µM

Figure 1: Anti-proliferative properties of A009.
HUVE cells were plated at low density (1000 cells/well) into 96-multiwell plates in M199 complete medium and treated with increasing dilutions of A009, hydroxytyrosol alone (HT) or ethanol (data not shown). Cell proliferation was evaluated at determined times (24, 48, 72, 96h) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 009 inhibited cell proliferation in a dose-dependent manner; the inhibitory effect of A009 was already significant after 24h of treatment with a dose of 1:500, whereas HT alone showed no significant inhibitory effect (A). We calculated the IC50 treatment in order to define the effectiveness of A009 and HT in inhibiting HUVE cells proliferation. A009 showed lower IC50 value as compared to HT alone, demonstrating the grater effectiveness of the purified extract (B).
Mean ± SD of 3 independent experiments is shown. Student's t-test was used to determine pvalue (***$p<0.001$; ****$p<0.0001$).

A009 inhibits HUVECs migration and invasion

Endothelial cells must cross basement membranes to form new blood vessels during angiogenesis. We therefore investigated whether the A009, HT or EtOH were able to affect HUVEC migration and invasion through matrigel, a reconstituted basement membrane [28,29]. A009, at 1:500 and 1:250 dilutions, significantly decreased the number of migrated (Figure 5A, $p<0.01$) and invaded (Figure 5B, $p<0.0001$) endothelial cells in a dose-dependent manner. A significant migration and invasion inhibitory effect with HT was observed only at the highest concentration.

A009 inhibits *in vivo* angiogenesis

We assessed the effect of A009 and HT on *in vivo* angiogenesis using a rapid and quantitative subcutaneous matrigel sponge assay [31]. A cocktail of VEGF, TNF-α and heparin promoted an hemorrhagic

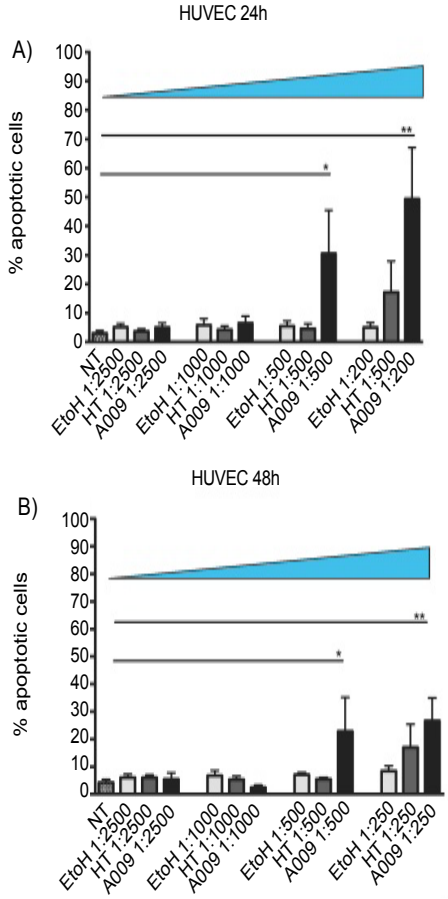


Figure 2: Induction of apoptosis by A009.
In the apoptosis assay, HUVECs (1.5×10^5 cells/well) were plated onto six-well plates and were treated with decreasing dilutions (1:2500-1:250) of A009 or HT for 24h (A) and 48h (B) in complete M199 medium. Vehicle (ethanol) and M199 medium alone were used as negative and positive controls, respectively. Fluorescence signal for Annexin V and 7 AAD (7-amino-actinomycin D) was used to detect viable, dead or apoptotic cells; the fluorescence signal was measured with a FACSCanto flow cytometer and data were analyzed with the FACSDiva Software 6.1.2. After 24h of treatment with A009, HUVECs underwent apoptosis in a dose-dependent manner, while HT induced apoptosis only at the highest concentration (1:250). Mean ± SD of 3 independent experiments is shown. Student's t-test was used to determine p-value. (*$p<0.05$ **$p<0.01$)

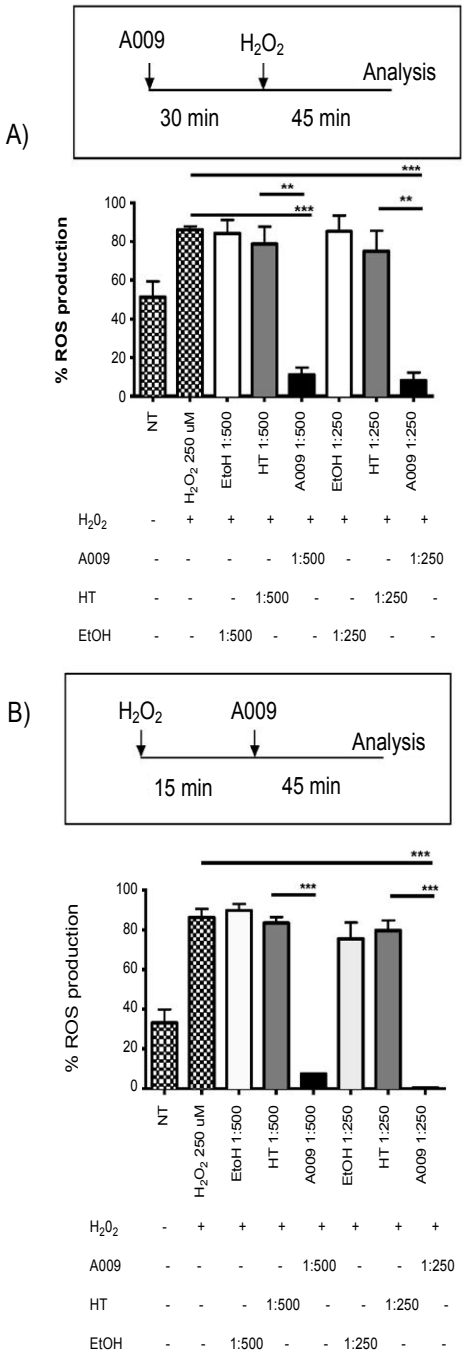


Figure 3: Anti-oxidative effects of A009
The ability of A009 to interfere with the induction of reactive oxygen species (ROS) was assessed by staining with DCFH-DA followed by flow cytometry analysis. HUVECs (1.5 x 10^5 cells/treatment) were pre-treated with decreasing dilution (1:500 and 1:250) of A009, HT and EtOH, then followed by exposure to 250μM H_2O_2 (A) or pretreated with 250μM H_2O_2 followed by treatment with decreasing dilution (1:500 and 1:250) of A009, HT and EtOH (B), to assess the scavenger activity in a prevention and an intervention schedule, respectively. Fluorescence was measured with FACSCanto and data analyzed with the FACSDiva Software 6.1.2. A009 was able to inhibit the ROS production in both schedules in a dose-dependent manner with more efficacy than HT alone. Complete medium (NT, medium M199 supplemented with 10% FBS) and H2O2 (250μM) were used as negative and positive control, respectively. Mean ± SD of 3 independent experiments is shown. Student's t-test was used to determine p-value. (**p<0.01;***p<0.001).

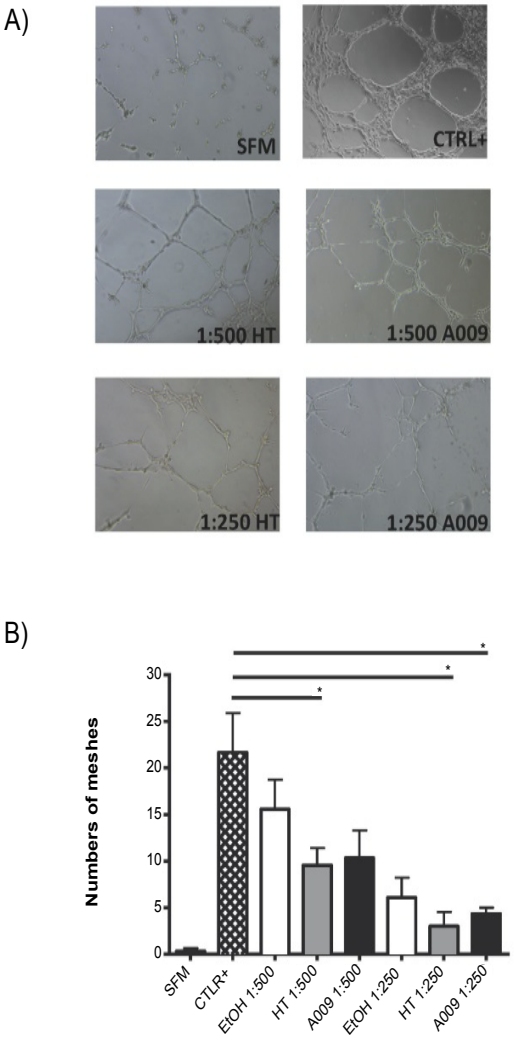


Figure 4: Effects of A009 on endothelial cell morphogenesis.
The anti-angiogenic activity of A009 and HT alone were tested in HUVECs using a morphogenesis assay. HUVECs (5 × 10^4 cells/well) were plated on a 24-multiwell plate,precoated with 10 mg/ml extracellular membrane basement matrix (Matrigel), then treated for 6 hours with decreasing dilutions (1:500-1:250) of A009, HT (A) or EtOH (data not shown) in complete M199 medium. Complete medium (CTRL+, M199 supplemented with 10% FBS) and serum-free medium (SFM) were used as positive and negative controls, respectively. The number of meshes were quantified with the ImageJ software "Angiogenesis Analyzer" tool (B). A009 and HT were both able to inhibit morphogenesis in an equivalent manner. Mean ± SD of 3 independent experiments is shown. Student's t-test was used to determine p-value (*p<0.05)

vascularization of the matrigel sponge, which was detectable at 4 days post-implantation. Addition of A009 (1:500 and 1:250) significantly inhibited the VTH-induced angiogenic response (1:500 p=0.0058 and 1:250 p=0.0189) as detected by macroscopic inspection of the pellets (Figure 6A). Quantification using the colorimetric Drabkin's assay for hemoglobin indicated a statistically significant inhibition of angiogenesis by A009 (Figure 6B). Interestingly, HT showed limited effects on angiogenesis *in vivo*. Hematoxylin and eosin stained sections of explanted-matrigel pellets confirmed the inhibition of angiogenesis process after A009 treatment (Figure 6C).

Discussion

There is a growing interest in finding novel active compounds

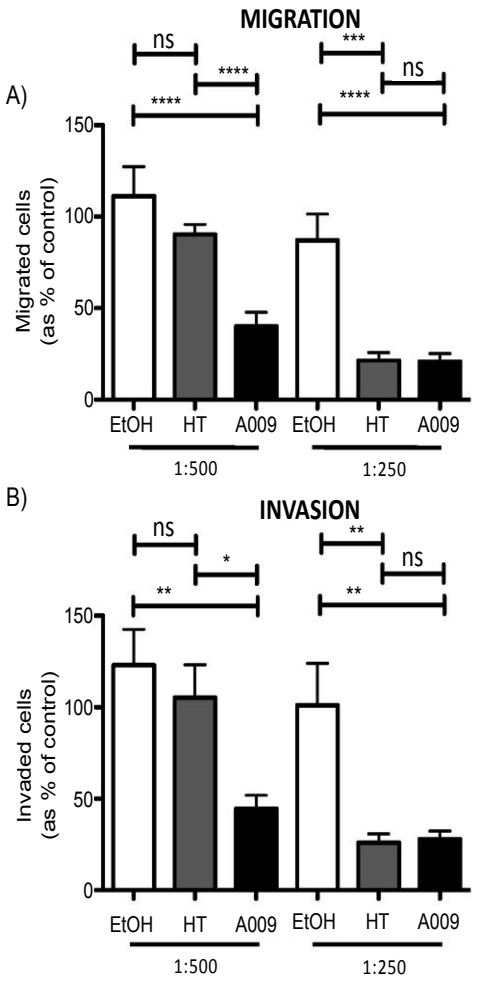


Figure 5: Effects of A009 on endothelial cell migration and invasion.
The ability of A009 to interfere with HUVEC migration (A) and invasion (B) were assessed using a modified Boyden chamber assays. HUVECs were pretreated for 24h with decreasing dilutions of A009, HT or EtOH (1:500-1:250), then the HUVECs were seeded in the upper compartment of Boyden chamber in serum-free medium. Complete medium (M199 supplemented with 10% FBS) or serum-free medium was placed in the lower compartment.
Following 6 or 24 hours of incubation for the assessment of migration or invasion, respectively, the migrated/invaded cells were fixed, stained and counted. A009 was able to inhibit migration and invasion at lower concentrations as compared to HT alone. Mean ± SD of 3 independent experiments is shown. Student's t-test was used to determine p-value (***p<0.001; ****p<0.0001) for migration and (*p<0.05; **p<0.01) for invasion.

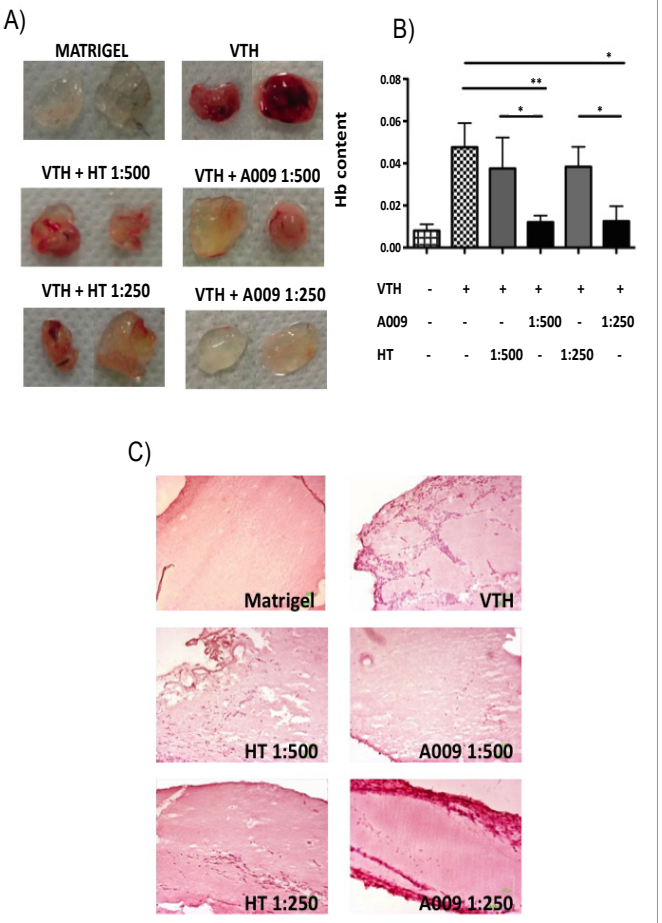


Figure 6: Anti-angiogenic activities of A009 *in vivo*.
Matrigel containing a pro angiogenic mixture (VTH: VEGF, TNF-α, heparin), with or without either A009 or HT alone, were subcutaneously injected into the flanks of mice. The Matrigel pellets were excised 4 days after injection, recovered, observed for macroscopic red coloration (A) and assessed for hemoglobin (Hb) content using the colorimetric Drabkin's assay (B). Hematoxylin and eosin staining of explanted matrigel pellets (C) were performed to evaluate the presence of total cell infiltrate. A009 was more effective than HT alone in inhibiting the angiogenic process. Mean ± SD is shown, (*p<0.05; **p<0.01).

derived from food and beverage sources [32] in relation to their biological properties and potential health benefits. Increased attention has been addressed to polyphenolic compounds, as a consequence of their anti-proliferative, anti-oxidant and anti-angiogenic properties [12,33]. Olive oil is a basic feature of Mediterranean diet that has been associated with diverse health effects and several studies have investigated the bioactive properties of its components [22,34]. Soluble phenolics compounds present in the olive fruit are discarded during production of olive oil in the form of OMWW. Given the concerns associated to waste product management, some studies addressed their attention to OMWW, identifying several compounds contained in this waste product that have been reported to be rich in phenolics [19]. Regarding the chemical composition of OMWW, HT has been reported as a major component [14], which has been demonstrated to exert anti-oxidant, anti-inflammatory and anti-tumor effects [15-17]. We investigated the properties of a novel purified polyphenol-enriched extract of OMWW abundant in HT consistently with the previous literature data [14], but containing a more complex mixture than pure HT, named A009. We investigated whether A009 was able to interfere with angiogenesis *in vitro* and *in vivo*. The anti-angiogenic and angio-preventive potential of A009 was compared with that of HT at similar concentrations.

The A009 extract exerted greater inhibition of endothelial cell proliferation, migration and invasion. Inhibition of cell proliferation was associated with the induction of apoptosis in treated HUVECs. HT showed a similar effect as A009 on endothelial cell morphogenesis. Given the previously reported anti-oxidant activity of HT, we examined the ability of A009 to inhibit ROS generation, compared to HT alone. We found that A009 exerted a more potent ROS scavenger activity than that of HT alone, either in a preventive or in a protective schedule. A009 was able to decrease the generation of ROS as a pre-treatment

or when administered after reactive species induction through H_2O_2. These data clearly support the hypothesis of a significant scavenger activity exerted by A009. Both A009 and HT were able to interfere with the ability of HUVECs to form capillary-like structures *in vitro*.

Finally, using the matrigel sponge assay, we demonstrated that A009 inhibited angiogenesis *in vivo*, as indicated by the decreased hemoglobin content in plugs excised from treated mice. The anti-angiogenic activity of A009 was more potent *in vivo* compared to HT alone. Altogether, our data demonstrate that A009, a highly phenolic-rich extract from OMWW, shows a significant anti-angiogenic and angiopreventive potential.

The use of two different batches, characterized by a different phenolic composition, demonstrated that the A009 complex, independently from the minimal variation in composition, exerts a stronger anti-angiogenic effect compared to hydroxytyrosol alone. This feature suggests that the A009 mixture has additional components that are additive/synergistic as compared to commercially available hydroxytyrosol. The differences in batch composition indicate that further investigation of the phenolic components distinguishing the different batches could shed light on the specific activity of specific components and indicate which cultivars might produce a greater effect, as olive oil phenols have been found to vary between cultivars [35]. Interestingly, the 2 batches varied widely in the presence of secoiridoid derivatives (29% in batch 1 and 4.7% in batch 2; Table S1). Secoiridoid derivatives, in particular (-)-oleocanthal, has shown effects as a *c-met* inhibitor [36,37]. The *met* pathway represents an additional VEGF independent mechanism of tumor angiogenesis, and inhibition of met inhibits tumor growth that correlates with repression of angiogenesis [38]. Taken together, our data demonstrated the anti-angiogenic and angio-preventive activity *in vitro* and *in vivo* of a novel phenols enriched extract showing promising application for preventive approaches/strategies, recovering a waste material.

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