

Assessment of the antioxidant activity of an olive oil total polyphenolic fraction and hydroxytyrosol from a Greek *Olea europea* variety in endothelial cells and myoblasts

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Abstract. Olive oil (OO) constitutes the basis of the Mediterranean diet, and it seems that its biophenols, such as hydroxytyrosol (HT) may scavenge free radicals, attracting distinct attention due to their beneficial effects in many pathological conditions, such as cancer. To the best of our knowledge, this is the first study in which the functional properties of an OO total polyphenolic fraction (TPF) and pure HT were examined in order to determine their antioxidant effects at a cellular level in endothelial cells and myoblasts. The test compounds were isolated using a green gradient-elution centrifugal partition chromatography-based method that allows the isolation of large volumes of OO in a continuous extraction procedure and with extremely low solvent consumption. For the isolation

of HT, a combination of two chromatographic techniques was used, which is effective for the recovery of pure compounds from complex natural extracts. Moreover, TPF and HT exhibited potent free radical scavenging activity *in vitro*. The cells were treated with non-cytotoxic concentrations and their redox status [in terms of glutathione (GSH) and reactive oxygen species (ROS) levels] was assessed. TPF extract was less cytotoxic than HT, and the observed differences between the two cell lines used suggest a tissue-specific activity. Finally, flow cytometric analysis revealed that both TPF and HT improved the redox status by increasing the levels of GSH, one of the most important antioxidant molecules, in both endothelial cells and myoblasts, while the ROS levels were not significantly affected.

Introduction

Aerobic organisms are exposed to reactive oxygen species (ROS) due to aerobic metabolism. ROS, such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH^{*}), have coalescent chemical properties that confer reactivity to a number of biological targets (1). ROS are often associated with oxidative stress, a condition characterized by the imbalance between the production of free radicals and the endogenous antioxidant mechanisms in the favor of the first (1). ROS are capable of reversibly or irreversibly damaging biological macromolecules, such as nucleic acids, proteins, lipids and carbohydrates (1). Thus, oxidative stress may lead to the development of a number of pathological conditions, such as cancer, diabetes, obesity, and neurodegenerative and autoimmune diseases (2). However, ROS also serve as signaling molecules which regulate biological and physiological processes, such as the regulation of signaling pathways, gene expression and apoptosis (1). In living organisms, there is a wide range of endogenous defensive mechanisms against free radicals, including enzymatic and non-enzymatic antioxi-

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Abbreviations: ROS, reactive oxygen species; OOs, olive oils; H₂O₂, hydrogen peroxide; FA, fatty acid; MUFA/SFA, mono-unsaturated/saturated fatty acid ratio; GSH, glutathione; HT, hydroxytyrosol; EVOO, extra virgin olive oil; TPF, total polyphenolic fraction; T, tyrosol; OLEO, oleocanthal; OLEA, oleacein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TLC, thin layer chromatography; CPC, centrifugal partition chromatography; EtOH, ethanol; MeOH, methanol; ISTD, internal standard; RSC, radical scavenging capacity; ¹H-NMR, proton nuclear magnetic resonance; OD, optical density; Nrf2, nuclear factor (erythroid-derived-2)-like 2

Key words: biophenols, C2C12 cells, EA.hy926 cells, hydroxytyrosol, olive oil extract, oxidative stress, reactive oxygen species

dants (2). Aside from the endogenous mechanisms, an organism may also acquire antioxidant components through diet (3,4). Polyphenols, products of secondary metabolism in plants, are some of the most important antioxidants (5,6).

The entire vascular system is comprised of a monolayer of endothelial cells. Endothelial cell integrity is necessary to the maintenance of the vessel wall and circulatory function (7,8). Moreover, the endothelium regulates the homeostasis, immune and inflammatory responses (8). Oxidative stress may cause damage to the vascular endothelium due to the loss of its integrity, leading to senescence and its detachment into the circulation (9). Thus, it consists one of the most important factors of pathological conditions of vessels, including atherosclerosis and thrombosis (8). In addition, oxidative stress may cause damage to the endothelium through leukocyte adhesion (10,11). Moreover, the interplay between ROS and nitric oxide sets off a vicious circle which leads to further endothelial activation and inflammation (8).

Oxidative stress also occurs in muscle tissue. Specifically, during intense exercise, a high rate of O₂ consumption takes place in skeletal muscle that can cause incomplete O₂ reduction and electron leakage from the electron transfer chain, leading to ROS generation and oxidative stress. Moreover, exercise induces oxidative stress through xanthine oxidase mechanisms (12). Oxidative stress, in turn, results in muscle fatigue, cell damage and apoptosis (13,14).

The medicinal properties of *Olea europaea* (olive tree) and its main product, olive oil (OO), has been known since ancient times (15). OO is the most important component of the Mediterranean diet (16), characterized by the intake of plant foods, such as fruits, vegetables, nuts and seeds (17). Epidemiological evidence indicates an inverse association with proper adherence to this diet and death due to coronary artery disease and various types of cancer, such as urinary bladder cancer, suggesting a potential protective role of regularly consumed OO and its hydrophilic fraction physiologically excreted through urine (18-20). The results of *in vitro* studies further support this evidence by revealing a protective effect of OO against various types of cancer (21-24), including urogenital neoplasms (25-27), paving the way towards research for the development of novel anticancer strategies. It has been shown in human prostate cell cultures for example, that exposure to OO induces an antioxidant effect on benign prostatic hyperplasia-1 cells and a pro-oxidant effect on malignant cells, suggesting that OO may potentially be used for prostate cancer prevention (25). Furthermore, it has been reported that increased OO consumption raises the concentration of phenol glucuronide conjugates in a dose-dependent manner in human urine (28) and that extra virgin OO (EVOO) extract suppresses the migration and invasion of T24 human bladder cancer cells by significantly inhibiting proliferation/motility in a dose-dependent manner (26). These promising results have been replicated in another study showing that EVOO extract significantly inhibited the proliferation and clonogenic ability of T24 and 5637 human bladder cancer cells in a dose-dependent manner by blocking the cell cycle, and that EVOO modulated the chemotherapeutic toxicity in bladder cancer cells (27).

Over the past 25 years, the consumption of OO worldwide has increased by >30% due to its two key characteristics, namely its nutritional and organoleptic properties (16,29).

The nutritional properties of OO are provided by its fatty acid (FA) profile and high monounsaturated/saturated FA ratio (MUFA/SFA), as well as by its rich antioxidant content, particularly that of biophenols which are believed to play a role in the prevention of diseases (16,30). OO biophenols act as oxidation chain-breakers, reacting with free radicals and forming inactive radicals. Some of the most important biophenols found in OO with marked biological activities, are oleocanthal (OLEO), oleacein (OLEA), oleic acid, oleuropein and its derivatives, tyrosol (T) and hydroxytyrosol (HT) (31). These compounds have the ability to scavenge free radicals by donating them an electron or hydrogen atom or by chelating metals (16). Among the OO biophenols, HT has attracted distinct attention due to its potent antioxidant activity attributed mainly to its orthodiphenolic structure (32). It is mainly found in olive tree leaves, olive pulp and OO (33). It is worth mentioning that HT can also be synthesized endogenously in the human body, as a product of dopamine oxidative metabolism, known as 3,4-dihydroxyphenyl ethanol (DOPET) (34). HT has been shown to exert preventive effects in several pathological conditions, such as metabolic syndrome, neurodegenerative diseases and cancer (34).

The aim of the present study was to examine the antioxidant properties of a EVOO total polyphenolic fraction (TPF) rich in biophenols, from a Greek endemic variety of *Olea europaea*, grown on Mount Athos, as well as that of pure HT. It is worth noting, that to date, at least to the best of our knowledge, there are no studies available investigating the antioxidant properties of an OO TPF in cell cultures. The antioxidants effects were assessed at a cellular level, particularly in EA.hy926 endothelial cells and C2C12 myoblasts. The obtained results will provide important information on the possible use of the TPF as an antioxidant food supplement.

Materials and methods

Reagents and materials. *n*-Hexane, ethyl acetate, ethanol (etOH) and acetonitrile were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Methanol (MeOH), dichloromethane and sulfuric acid (H₂SO₄ >95%) were obtained from Fisher Scientific UK (Leicestershire, UK). All solvents were of analytical grade. Deionized water was used to prepare all aqueous solutions. Vanillin standard were purchased from Sigma-Aldrich (Poole, UK) with purity of >95%.

Extraction of TPF from EVOO. EVOO was procured from Northern Greece (Mount Athos) which was freshly produced in January 2015). The extraction of TPF was achieved following a previously described procedure (35). Liquid-liquid extraction was carried out on a laboratory-scales Fast Centrifugal Partition Extractor FCPE300[®] (Rousselet-Robatel Kromaton, Anonay, France; column capacity of 300 ml) connected to a LabAlliance preparative pump. The extraction process consisted of several 'Extraction-Recovery' cycles (multi-dual-mode method) using as 'mobile phase' a mixture of *n*-hexane/OO in proportion of 3:2 (v/v) and as 'stationary phase' etOH/water in proportion of 3:2 (v/v). The procedure starts by filling the CPE column with 0.3 liters of the stationary phase in ascending mode and at 200 rpm. The rotation speed was increased up to 1,000 rpm and the mobile phase (feed oil phase) was pumped

at 60 ml/min in ascending mode. After passing 2.5 liters of mobile phase (extraction step) the pumping mode was switched to 'descending' and a volume of 0.3 liters of aqueous extraction phase was pumped (at the same flow rate and rotation speed) in order to replace the concentrated in biophenols stationary phase with fresh one (recovery step). This cycle of extraction-recovery was repeated 5 more times extracting totally 15 liters of feed oil phase corresponding to 6 liters of OO. The 6 collected stationary phases were evaporated under a vacuum and the obtained viscous extract was defatted using the biphasic system *n*-hexane/ acetonitrile (1/1 v/v) and stored at 4°C for further use. Finally, the extraction of 6 liters of OO resulted in the recovery of 6.35 g of OO biophenol extract.

Purification of HT from TPF. High purity HT was recovered from the treatment of TPF in a two steps separation process. Initially, the extract was fractionated by gradient-elution centrifugal partition chromatography (CPC) and the enriched in the HT fraction was further analyzed by using Sephadex LH-20 (Sigma-Aldrich) column chromatography. The CPC experiment was performed on a FCPC1000[®] apparatus (Rousset-Robatel Kromaton) equipped with a preparative column (1,000 ml total column capacity) and connected to a LabAlliance Preparative pump. The system was coupled to a SPECTRA SYSTEM UV 2000 detector set at 254, 280 and 366 nm (Thermo Fisher Scientific, Inc., Waltham, MA, USA), while fractions were collected using a Büchi B-684 fraction collector (Büchi Labortechnik AG, Flawil, Switzerland).

The TPF was fractionated in a step-gradient elution mode following a previously described method (35). For this purpose, a series of 4 biphasic systems consisting of the solvents, *n*-hexane, ethyl acetate, EtOH, and water in proportions 4/1/2/3 (S1), 3/2/2/3 (S2), 2/3/2/3 (S3) and 1/4/2/3 v/v/v/v (S4) were used. The solvents were thoroughly mixed in a separating funnel at room temperature prior to use, and the 2 phases of each system were separated after equilibration of the mixture. The column was initially filled with the lower phase of the first biphasic system (stationary phase) in ascending mode at a flow rate of 30 ml/min and 200 rpm. The rotation speed was increased to 1,000 rpm and the mobile phase I (upper phase of S1) was pumped at a flow rate of 15 ml/min. When the hydrodynamic equilibrium of the two liquid phases inside the CPC column was established (Sf=67%), 5 g of sample (diluted in 20 ml of lower and 10 ml of upper phase of S1) was injected into the column via a 30 ml sample loop. A sequential pumping of the upper mobile phases of S1, S2, S3 and S4 was then performed in volumes of 500, 1,100, 1,400 and 1,000 ml, respectively (gradient elution step). The experiment completed by pumping the stationary phase in ascending mode (at the same flow rate and rotation speed) and collection of 30 more fractions (extrusion step). Fraction collector was set to collect 25 ml fractions during the all experiment.

All collected fractions were evaluated using thin layer chromatography (TLC). The plates were coated (Merck Millipore, Billerica, MA, USA) with silica gel 60 F254 and developed in dichloromethane/MeOH in different proportions. After detection at UV254 and UV366, the plates were sprayed with vanillin-sulfuric acid and heated. Based on the TLC qualitative results, the fractions containing HT (195 fractions of 25 ml) were combined and evaporated to dryness resulting to 95 mg of

an enriched HT fraction (86% purity). For further purification, Sephadex LH-20 column chromatography was incorporated.

Specifically, 90 mg of the sample (diluted in 1 ml EtOH) were subjected to column chromatography (Sephadex[®] LH-20, 10x285 mm) and eluted with EtOH. All obtained fractions were initially analyzed by TLC and those of similar chemical content were combined giving finally three main fractions (Fractions A-C). Fraction B contained 71.3 mg HT in high purity (>97%). The purity was determined by quantitative high-performance liquid chromatography (HPLC) analysis.

HPLC-diode-array detection (DAD) qualitative and quantitative analysis. The qualification analysis of TPF and quantification of HT, T, OLEO and OLEA were performed on a Thermo Finnigan HPLC instrument (Ontario, Canada) equipped with a SpectraSystem P4000 pump, a SpectraSystem 1000 degasser, a SpectraSystem AS3000 automated injector, and a UV SpectraSystem UV6000LP detector monitored at 235, 280 and 365 nm. The samples were analyzed in a Discovery HS C18 (15cm x 4.6mm, particle size 5 µm) analytical column (Supelco, Bellefonte, PA, USA) using two different methods. The first one (method A) was used for the qualification analysis of the stable TPF components and quantification of HT and T, and was based on a previously published method with minor modifications (36). Water acidified with 0.2% acetic acid (solvent A) and acetonitrile (solvent B) was used as a solvent in the following gradient system: 0-40 min 2-30% B, 40-45 min 30% B, 45-50 min 30-2% B. The flow rate was set at 1 ml/min, the injection volume was 20 µl (from a solution of 1 mg TPF/ml MeOH) and the total run duration was 50 min. The qualification analysis of the labile TPF components and quantification analysis of OLEA and OLEO was performed using the second method (method B). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), while the gradient elution began with 80% of solvent A decreasing to 70% in 20 min, remaining stable for 15 min and increasing again to 80% in the next 5 min (total duration, 40 min). The flow rate was set at 1 ml/min, while the injection volume was 20 µl (from a solution of 1 mg TPF/ml MeOH), as previously described (37,38).

The quantification analysis of HT and T was performed using method A. The calibration curves were created using the concentrations 2, 3, 20, 50 and 100 µg/ml and was used to calculate the percentage of the target compounds in EVOO and TPF, as well as to examine the purity of HT both in CPC fraction and in the final pure form. The peak area values (measured at 280 nm) constitute the average of 3 measurements. Linear regression analysis with the use of syringaldehyde (98% HPLC; ExtraSynthese, Genay Cedex, France) as an internal standard (ISTD) was performed.

The quantification of OLEO and OLEA was performed using method B. The calibration curves were constructed using 10 concentrations levels (50,100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 µg/ml). ISTD was prepared in a mixture of MeOH/water 1:1 (v/v) at a concentration of 1 mg/ml and stored at -4°C. Both compounds are eluted, as double peaks and thus the quantification was based on the total area of both peaks (measured at 235 nm) as previously described by Impellizzeri and Lin (37). In the case of HT and T, the quantification was based on the ratio between the peak area of the analyte

and the peak area of ISTD. The equations and the coefficient of determination (R^2) of the standard curves were as follows: $y=45130x+22444$, $R^2=0.995$ for OLEO, $y=43653x-1E+06$, $R^2=0.997$ for OLEA, $y=0.041x-0.011$, $R^2=0.999$ for HT and $y=0.025x-0.014$, $R^2=0.999$ for T.

TLC and NMR analysis. The TLC analysis of TPF and CPC fractions was performed on Merck 60 F254pre-coated silica gel plates and developed with DCM/MeOH 95:5 (v/v). The TLC chromatograms were firstly revelation by using UV light at 254 and 365 nm and the TLC plates were then sprayed by a vanillin (5% w/v in ethanol)- H_2SO_4 (50% v/v in methanol) solution and heated at 100-120°C for 2-3 min. Structure confirmation of the isolated HT was achieved by NMR analysis. The 1H -NMR experiments were performed on a 600 MHz on a Bruker AvanceAVIII-600 spectrometer (Karlsruhe, Germany) equipped with a TXI cryoprobe (Wisssembourg, France) in CD3OD.

Assessment of the total polyphenolic content (TPC) of the extracts. The TPC of the OO extract was determined using the Folin-Ciocalteu reagent, as previously described (39). A total of 20 μ l of extract were added to a tube containing 1 ml distilled water, followed by the addition of 100 μ l of Folin-Ciocalteu reagent and incubation for 3 min at room temperature. Subsequently, 280 μ l of 25% w/v sodium carbonate solution along with 600 μ l of distilled water were added to the mixture. Finally, following 1 h incubation at room temperature in the dark, the absorbance was measured at 765 nm versus a blank lacking the extract. The measurement was carried out on a Hitachi U-1900 radio beam spectrophotometer (serial no. 2023-029; Hitachi, Ltd., Tokyo, Japan). The optical density of the sample without the Folin-Ciocalteu reagent at 765 nm was also measured. The TPC was determined using a gallic acid standard curve (50-1,500 μ g/ml). The TPC is presented as μ g of gallic acid equivalents per mg of extract.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The free-radical scavenging capacity (RSC) of the TPF and HT extracts was evaluated using the DPPH radical assay (40). Briefly, a 1 ml freshly prepared methanolic solution of DPPH radical (100 μ M) was mixed with the tested extract solution at various concentrations (7.8-250 μ g/ml TPF and 0.7-100 μ g/ml HT). The mixture was vortexed and incubated at room temperature in the dark for 20 min, followed by absorbance measurement at 517 nm on a Hitachi U-1900 radio beam spectrophotometer (serial no. 2023-029; Hitachi, Ltd.). MeOH was used as a blank and DPPH alone in MeOH was used as the control.

The percentage RSC of the tested extracts was calculated using the following equation: $RSC (\%) = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$, where $OD_{control}$ and OD_{sample} are the optical density (OD) values of the control and the test sample, respectively. Moreover, the IC_{50} value indicating the polyphenolic amount that caused 50% scavenging of the DPPH radical was calculated. In order to compare the radical scavenging efficiency of the extracts, the specific activity was determined. The specific activity was evaluated by dividing the IC_{50} value obtained from the DPPH and 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) assays by the amount of polyphenols contained

in each mg of the TPF and HT. All experiments were carried out in triplicate and on at least 3 separate occasions.

ABTS^{•+} radical scavenging assay. The ABTS^{•+} RSC of the extract was determined as previously described by Cano *et al.* (41) with minor modifications. Briefly, 1 ml reactions were prepared in distilled water containing ABTS^{•+} (1 mM), H_2O_2 (30 μ M) and horseradish peroxidase (6 μ M) in 50 mM phosphate-buffered saline (PBS; pH 7.5). The solution was vortexed, followed by incubation for 45 min at room temperature in the dark. Subsequently, 10 μ l of the tested extracts, at various concentrations, were added and the absorbance at 730 nm was read on a Hitachi U-1900 radio beam spectrophotometer (serial no. 2023-029; Hitachi, Ltd.). In each experiment, a blank lacking the peroxidase was used, while the ABTS^{•+} radical solution without the extract was used as the control. The RSC percentage and the specific activity values were determined as described above for the DPPH method. All experiments were carried out in triplicate and on at least 3 separate occasions.

Cell culture conditions. The C2C12 murine myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (all from Gibco, Paisley, UK) in plastic disposable tissue culture flasks at 37°C in 5% CO_2 (42). The C2C12 myoblasts were donated by from Professor Koutsilieris (National and Kapodistrian University of Athens, Athens, Greece).

EA.hy926 endothelial cells were cultured as described previously in tissue culture flasks at 37°C in 5% CO_2 (34,42). The medium used was DMEM, containing 10% (v/v) FBS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (Gibco) (42). The EA.hy926 cells were donated by Professor Koukoulis (University of Thessaly, Larissa, Greece).

XTT assay. Cell viability was assessed using the XTT assay kit (Roche, Mannheim, Germany) as described previously (42). Briefly, the C2C12 and EA.hy926 cells were cultured in a 96-well plate in a density of 1×10^4 cells per well in DMEM. Following 24 h of incubation, various concentrations of the TPF and HT extracts in serum-free DMEM were administered for 24 h. Afterwards, 50 μ l of XTT test solution were added to each well. Following 4 h of incubation, absorbance was measured at 450 nm and also at 630 nm as a reference wavelength in a BioTek ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Serum-free DMEM was used as a negative control. In addition, the absorbance of the TPF and HT alone in serum-free DMEM and XTT test solution was measured at 450 nm. The absorbance values of the TPF and HT alone were subtracted from those derived from cell treatment with the tested compounds. Data were calculated as the percentage of viability using the following formula: $Viability (\%) = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$, where $OD_{control}$ and OD_{sample} indicate the OD of the negative control and the tested compounds, respectively. All experiments were carried out in triplicate and on 3 separate occasions.

Assessment of GSH and ROS levels in cells by flow cytometry. The intracellular GSH and ROS levels were assessed using the

fluorescent dyes, mercury orange and 2,7-dichlorofluorescein diacetate (DCF-DA), respectively (43). Mercury orange binds directly to GSH, while DCF-DA is deacetylated within cells by esterases and is further converted to fluorescent DCF by the oxidative action of ROS. A 400 μM stock solution of mercury orange was prepared in acetone and stored at 4°C, and a fresh 400 μM stock solution of DCF-DA was prepared in MeOH. To measure the GSH and ROS levels the cells were first trypsinized and centrifuged at 300 \times g for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in PBS at 1×10^6 cells/ml and incubated in the presence of mercury orange (40 μM) or DCF-DA (10 μM) in the dark at 37°C for 30 min. The cells were then washed, resuspended in PBS, and subjected to flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with excitation and emission wavelengths at 488 and 530 nm for ROS and at 488 and 580 nm for GSH, respectively. In addition, forward-angle and right-angle light scattering showing the cell size and cell internal complexity, respectively, were measured. The cells were analyzed at a flow rate of 1,000 events/sec. Analyses were performed on 10,000 cells per sample, and the fluorescence intensities were measured on a logarithmic scale. Data were analyzed using BD Cell Quest software (BD Biosciences). Each experiment was repeated at least 6 times.

Statistical analysis. All results are expressed as the means \pm standard error of the mean. A Spearman's correlation analysis for examining the results from the TPC, DPPH and ABTS⁺ assays was performed. A value of $P < 0.05$ was considered to indicate a statistically significant difference. In addition, one-way ANOVA was applied, followed by Dunnett's tests for multiple pair wise comparisons using SPSS software (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Preliminary analysis by our research group on different EVOOs demonstrated that EVOO originating from the olive tree variety grown in Mount Athos is rich in biophenols (data not shown). Thus, this EVOO was used for the isolation of the TPF extract, as well as that of pure HT. TPF extract was also characterized chemically by determining the amount of HT, T, OLEO and OLEA biophenols. Moreover, both TPF and HT were examined for their antioxidant activity *in vitro* and at a cellular level in endothelial cells and myoblasts.

The recovery of the TPF was performed by applying a recently developed CPE-based extraction process which included the use of the biphasic system *n*-hexane/EVOO/etOH/water-3/2/3/2 v/v/v/v and a multiple dual-mode CPE method for the treatment of OO (27,35). Compared to other purposed OO extraction methods (44,45), this green process allows the treatment of large volumes of OO in a continuous extraction procedure and with extremely low solvent consumption. Thus, 6 extraction-recovery cycles were performed by pumping per each cycle 2.5 liters of feed oil phase (at 60 ml/min in ascending mode) and replacing the concentrated in biophenols stationary phase with 300 ml of fresh aqueous extraction phase (see experimental part). It is important to note that the treatment by this process of 6 liters

of OO (corresponding to 5,478 g) resulted the recovery of 6.35 g of TPF in only 5 h. However, due to the high complexity of TPF the first step of the separation process included a step-gradient CPC fractionation with a series of four biphasic systems consisting of the solvents *n*-hexane, ethyl acetate, etOH and water in proportions 4/1/2/3 (S1), 3/2/2/3 (S2), 2/3/2/3 (S3) and 1/4/2/3 v/v/v/v (S4) (27,35). Even if this method afforded approximately HT in 87% purity, an additional purification step was required and therefore size exclusion column chromatography was used. The combination of these two chromatographic techniques has the advantage of using different criteria of selectivity between two purification steps and has been successfully applied for the recovery of pure compounds from complex natural extracts (46). The result of this two-step purification procedure was the effective recovery of high purity (>97%) HT. The purity of the recovered compounds was firstly examined by proton nuclear magnetic resonance (¹H-NMR) and was then evaluated by HPLC-DAD analysis.

In addition, since the compounds under investigation behave differently in the presence or absence of acid two different elution systems have been used. Specifically, for the analysis of the TPF constituents, such as the phenyl alcohols (HT and T), the mobile phase of the used HPLC method (method A, see Materials and methods) contained acidified solvent (water acidified with 0.2% acetic acid) in order to increase the quality of the analysis. On the other hand, OLEA and OLEO are labile components and they are not stable in acidic conditions. Thus, the mobile phase of the HPLC method (method B, see Materials and methods) used for the analysis of those compounds was composed of water (solvent A) and acetonitrile (solvent B) without acidification. The profiling of TPF as resulting from HPLC-DAD chromatograms is depicted in Fig. 1. The results of the quantitative analysis are expressed in mg/g TPF. Specifically, TPF extract contained 19.4 mg/g HT, 24.4 mg/g T, 56.3 mg/g OLEA and 135.5 mg/ml OLEO.

As indicated by the quantitative analysis, the TPF was rich in biophenols. Specifically, the TPC as measured by Folin-Ciocalteu assay was 484 μg of biophenols per mg of TPF extract. According to a comparative study in which 221 EVOOs were extracted from 4 olive mono-cultivars (Koroneiki, Tsounati, Adramitini, and Throubolia) originating from 4 divisions of Greece (47), the range of TPC was between 30-351 mg/kg EVOOs. Of note, according to a health claim on OO polyphenols approved by the European Food Safety Authority (EFSA; Commission Regulation EU 432/2012), OOs are considered to protect from oxidative stress-induced lipid peroxidation in blood, when they contain about 5 mg of HT and its derivatives (e.g., oleuropein complex and T) per 20 g of OO. Based on the content of TPF in biophenols, OO in the present study, contain 4.15 mg of HT and its derivatives per 20 g.

The chemical analysis of TPF indicated that the T levels were high (28.28 mg/kg TPF) and close to those of HT (22.48 mg/kg TPF), according to a comparative study, where the T levels fluctuated between 1.97-16.2 mg/kg OO (47). Additionally, OLEO exhibited the highest concentration which was >2-fold higher than that of OLEA.

Following the determination of the biophenolic content, the antioxidant activities of TPF and HT were evaluated by DPPH and ABTS⁺ assays (Fig. 2). In order to examine the antioxidant potency of the biophenols contained in the tested compounds,

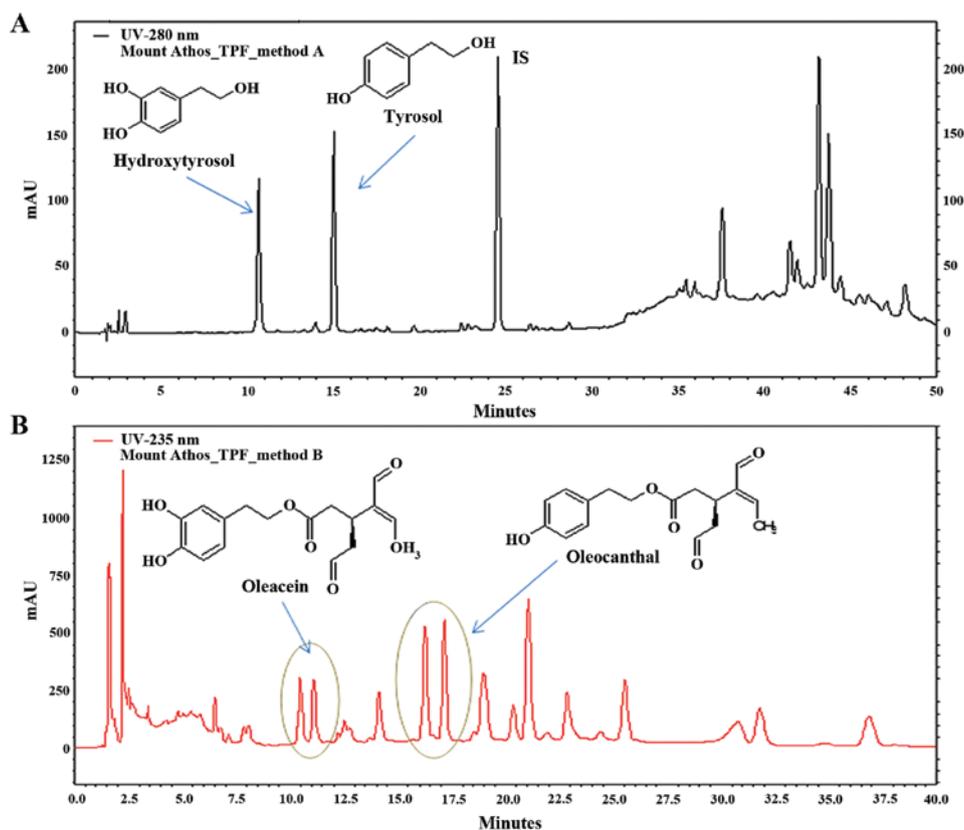


Figure 1. HPLC-DAD chromatograms of TPF. (A) Chromatogram recorded with method A suitable for the analysis of HT and T. (B) Chromatogram recorded with HPLC method B suitable for the analysis of OLEA and OLEO. OLEA and OLEO are eluted, as double peaks. HPLC-DAD, high-performance liquid chromatography-diode-array detection; TPF, total polyphenolic fraction; HT, hydroxytyrosol; T, tyrosol; OLEA, oleacein; OLEO, oleocanthal.

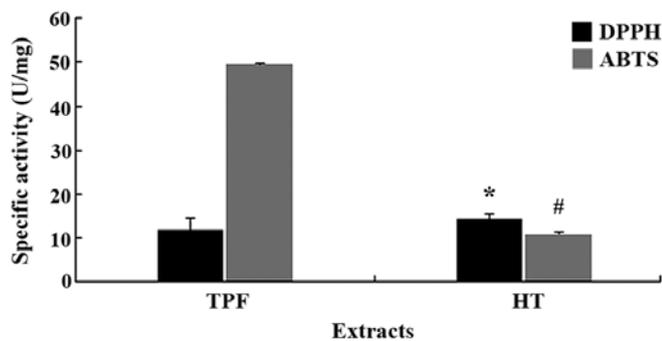


Figure 2. Antioxidant activity as assessed using DPPH and ABTS^{•+} assays. The specific activity of each test compound is shown, as measured in units of activity per mg of TPF and HT. A unit represents the amount of biophenols required to scavenge 50% of DPPH and ABTS^{•+} radicals. The TPF concentrations expressed as polyphenol content divided to TPC. All data are expressed as the means \pm standard error of the mean of 3 experiments (n=3). *P<0.05 indicated a statistically significant difference between TPF and HT in the DPPH method and #P<0.05 in the ABTS^{•+} method. TPF, total polyphenolic fraction; HT, hydroxytyrosol.

the specific activity was determined. Specific activity is a unit that compares extracts with varying composition. Since HT was a pure polyphenol, for the calculation of the specific activity, its polyphenolic content was considered as 100%. According to DPPH assay, HT exhibited a significantly ($P<0.05$) higher antioxidant activity compared with TPF. by contrast, in ABTS^{•+} assay, the antioxidant activity of HT was significantly ($P<0.05$) lower than that of TPF. These differences between the DPPH and

ABTS^{•+} assays may be ascribed to the different solvents used in each assay (48). In particular, in DPPH assay the solvent is MeOH while in ABTS^{•+} assay the solvent is water (49). Namely, the bioactive compounds of TPF may be more polar and so they were dissolved more in water and less in MeOH than HT.

The antioxidant activity of the tested compounds was also examined in C2C12 myoblasts and EA.hy926 endothelial cells. In order to use non-cytotoxic concentrations of the tested compounds for these experiments, their cytotoxicity was assessed by XTT assay. The results from XTT assay revealed that TPF exhibited cytotoxicity at concentrations $>24.0 \mu\text{g/ml}$ in both the C2C12 and EA.hy926 cells (Fig. 3A and B). HT exerted cytotoxic effects at concentrations $>30.0 \mu\text{g/ml}$ in the C2C12 cells and $>2.5 \mu\text{g/ml}$ in the EA.hy926 cells (Fig. 3C and D). Thus, the range of concentrations used for assessing the antioxidant activity of the tested compounds were 2.5-10.0 $\mu\text{g/ml}$ for TPF in both the C2C12 and EA.hy926 cells, and 10-25 and 0.25-1.0 $\mu\text{g/ml}$ for HT in the C2C12 and EA.hy926 cells, respectively.

The results from flow cytometric analysis revealed that treatment of the C2C12 cells with TPF significantly increased the GSH levels by 26, 28, 37 and 23% at 2.5, 5, 7.5 and 10 $\mu\text{g/ml}$, respectively, compared with control (Fig. 4A). Treatment of the C2C12 cells with HT also significantly increased the GSH levels by 24, 23, 54 and 38% at 10, 15, 20 and 25 $\mu\text{g/ml}$, respectively (Fig. 4B). Moreover, treatment of the EA.hy926 cells with TPF and HT significantly increased the GSH levels compared to the controls (Fig. 4C and D). Thus, the effects of TPF and HT on the GSH levels seemed to be cell type-independent. Particularly,

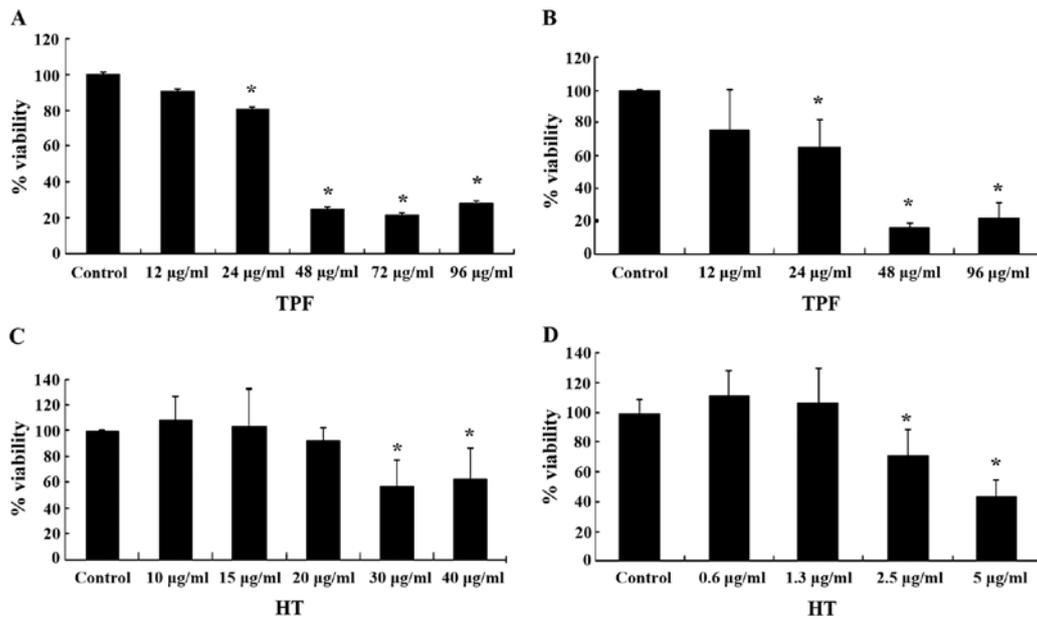


Figure 3. Assessment of the effects of tested compounds on the viability of C2C12 myoblasts and EA.hy926 endothelial cells after 24 h of treatment by XTT assay. (A) Cytotoxicity of TPF in C2C12 cells. (B) Cytotoxicity of TPF in EA.hy926 cells. (C) Cytotoxicity of HT in C2C12 cells. (D) Cytotoxicity of HT in EA.hy926 cells. The TPF concentrations expressed as polyphenol content divided to TPC. All data are expressed as the means \pm standard error of the mean of 6 experiments (n=6). *P<0.05 indicated a statistically significant difference compared to the control. TPF, total polyphenolic fraction; HT, hydroxytyrosol; TPC, total phenolic content.

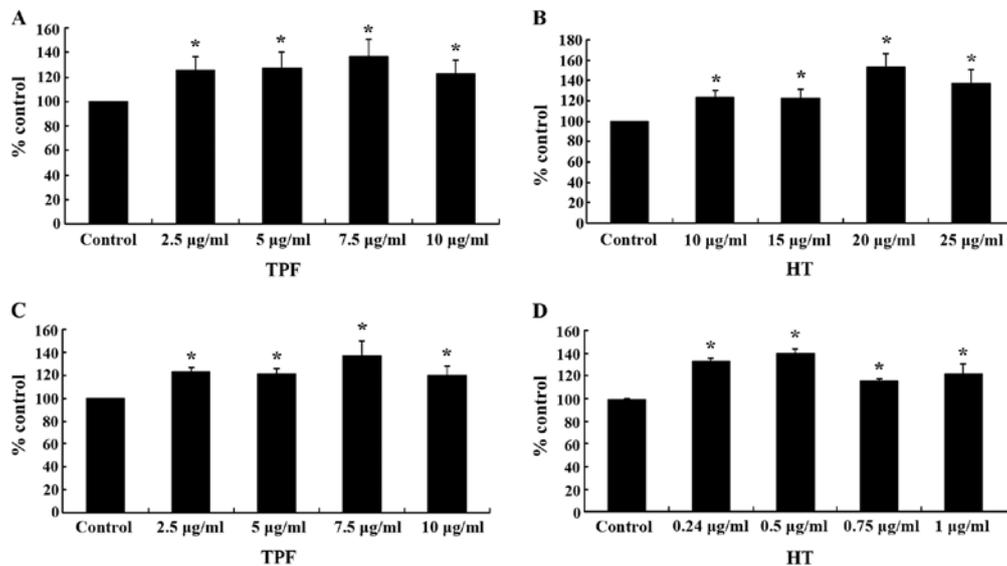


Figure 4. Effects of the test compounds on GSH levels in C2C12 myoblasts and EA.hy926 cells after treatment for 24 h, as assessed by flow cytometry. (A) Effects of TPF extract on C2C12 cells. (B) Effects of HT on C2C12 cells. (C) Effects of TPF extract on EA.hy926 cells. (D) Effects of HT on EA.hy926 cells. Bar charts showing the GSH levels, as calculated by BD Cell Quest software. The TPF concentrations expressed as polyphenol content divided to TPC. All results are expressed as the means \pm standard error of the mean of 6 experiments (n=6). *P<0.05 indicated a statistically significant difference between TPF or HT concentrations and the control. TPF, total polyphenolic fraction; HT, hydroxytyrosol; GSH, glutathione; TPC, total phenolic content.

TPF increased the GSH levels in the EA.hy926 cells by 23, 22, 38 and 20% at 2.5, 5, 7.5 and 10 $\mu\text{g/ml}$, respectively, while HT by 34, 40, 15 and 22% at 0.25, 0.5, 0.75 and 1.0 $\mu\text{g/ml}$ respectively, compared to controls (Fig. 4C and D). The HT-induced increase in GSH levels suggested that this polyphenol was one of the bioactive compounds in TPF accounting for its effects on the GSH levels.

HT has been shown to increase GSH synthesis in human retinal pigment epithelial cells by the activation of the

nuclear factor (erythroid-derived-2)-like 2 (Nrf2), a transcription factor and a key regulator of the antioxidant defense system (Fig. 6) (50-53). Nrf2 induces the expression of gamma-glutamylcysteine ligase (GCL), which has the ability to regulate the synthesis of GSH (54). Moreover, HT seems to activate the NK-p62/SQSTM1 pathway, required for Nrf2-induced GSH synthesis (42,50). Manzanares *et al* (55) indicated that after a high EVOO diet, the activation of Nrf2 was observed in rat livers and mammary glands.

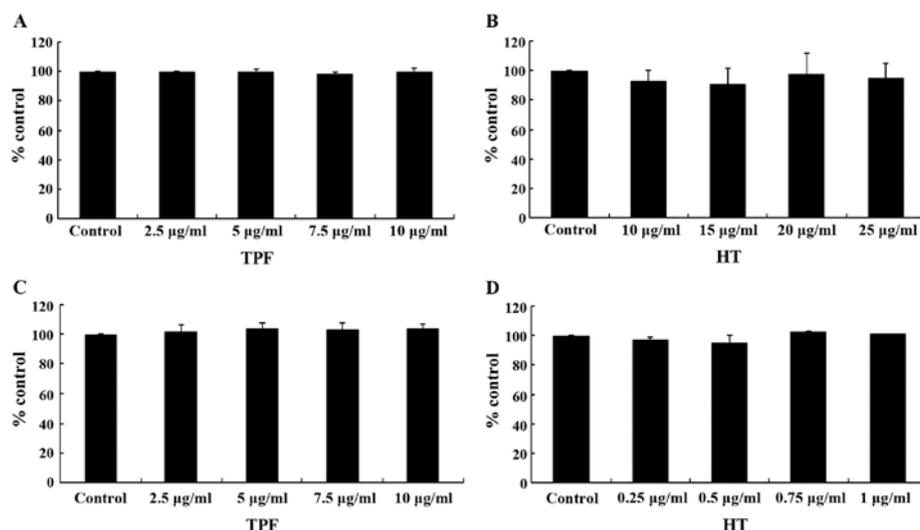


Figure 5. Effects of the test compounds on ROS levels in C2C12 myoblasts and EA.hy926 cells after treatment for 24 h, as assessed by flow cytometry. (A) Effects of TPF extract on C2C12 cells. (B) Effects of HT on C2C12 cells. (C) Effects of TPF extract on EA.hy926 cells. (D) Effects of HT on EA.hy926 cells. Bar charts showing the ROS levels, as calculated by BD Cell Quest software. All results are expressed as the means \pm standard error of the mean of 6 experiments (n=6). *P<0.05 indicated a statistically significant difference between TPF or HT concentrations and the control. The TPF concentrations expressed as polyphenol content divided to TPC. TPF, total polyphenolic fraction; HT, hydroxytyrosol; ROS, reactive oxygen species; TPC, total phenolic content.

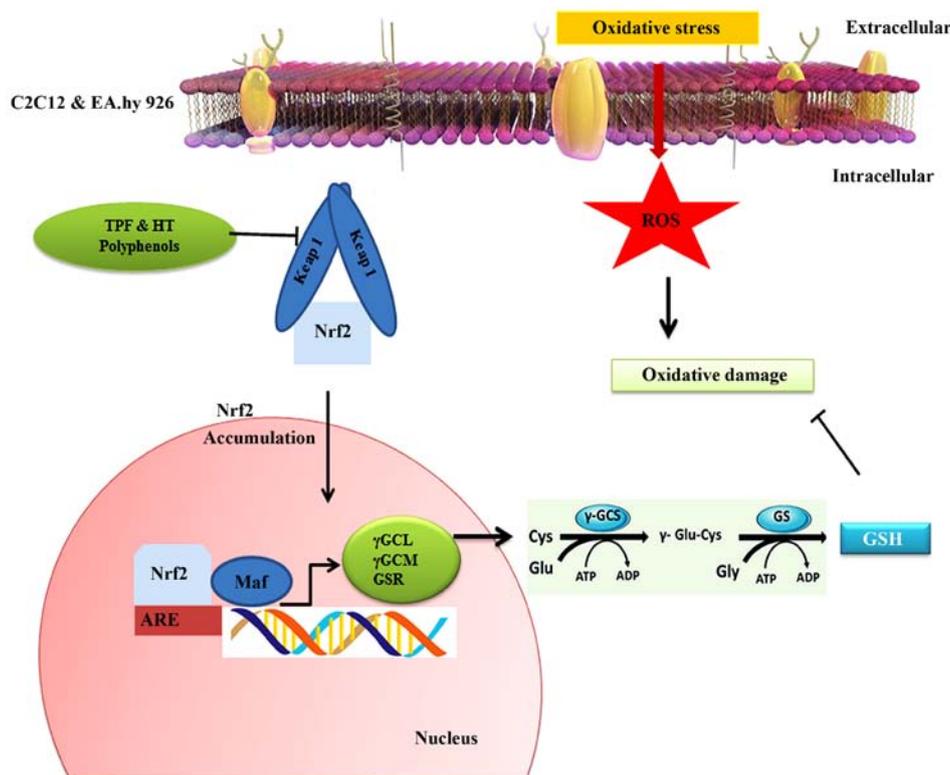


Figure 6. Possible mechanisms through which TPF and HT exert their effects on GSH levels. TPF, total polyphenolic fraction; HT, hydroxytyrosol; Nrf2, nuclear factor (erythroid-derived-2)-like 2; ROS, reactive oxygen species; GSH, glutathione; ARE, antioxidant response element.

Moreover, OO biophenols, from olive mill waste water have been shown to increase GSH concentrations in human blood (48,56). This increase in GSH levels may be due to the upregulation of GCL and GSH synthetase, mediated by the antioxidant response element (ARE; a target of Nrf2) (Fig. 6) (56). Covas *et al* indicated that an EVOO rich in biophenols (366 mg/kg) beneficially modulated the balance between GSH and oxidized

glutathione (GSSG). Thus, EVOO biophenols exert their antioxidant effects as a part of the induction of Nrf2 (57).

It is also worth mentioning that at the highest concentration, both TPF and HT exhibited less potency for increasing the GSH levels than at lower concentrations. This observed decrease in the potency of TPF and HT at the highest concentrations may be explained by a potential pro-oxidant activity of polyphenols

after reaching a 'crucial' concentration (43,58-61). A potential pro-oxidant activity was also supported by the cytotoxicity exerted by TPF and HT after these high concentrations.

Oleuropein and HT have been reported to exert pro-oxidant effects, due to their iron- and copper-reducing activities. These reduced metals, in turn, can catalyze the production of OH[•] radicals by the Fenton reaction. The ability of dietary polyphenols to act as antioxidants/prooxidants under *in vitro* and *in vivo* systems is dependent on a number of factors such their concentration and structure (62).

The observed TPF- and HT-induced increase in GSH levels in C2C12 myoblasts and EA.hy926 endothelial cells is of importance. GSH is a conserved molecule among many species, reflecting its crucial biological role. In particular, it has been established that the thiol moiety of GSH is important for the direct scavenging of radical species (63). Thus, a decrease at GSH levels contributes to oxidative stress associated with aging and many pathological conditions, such as neurodegenerative diseases, inflammation, and infections (63).

Unlike GSH, the ROS levels were not significantly affected by the two test compounds compared to the controls in neither cell line (Fig. 5). It should be mentioned that the measured ROS levels corresponded to the naturally occurring levels in these cells; that is, there was not any treatment of cells with oxidizing agents before the addition of TPF and HT. Previous studies have also reported that changes in oxidative stress levels or antioxidant mechanisms are not always accompanied by changes in ROS levels (42,43,64).

In conclusion, EVOO from a Greek endemic olive tree variety, *Olea europea*, was used for the isolation of TPF, as well as that of pure HT. For the extraction process, it was used a recently developed green CPE-based method that allows the treatment of large volumes of OO in a continuous extraction procedure and with extremely low solvent consumption. For the isolation of pure HT, a combination of two chromatographic techniques was used, which has the advantage of using different criteria of selectivity between two purification steps and thus it is effective for the recovery of pure compounds from complex natural extracts. As indicated from the quantitative analysis, the TPF extract was rich in biophenols, such as T, HT, OLEO and OLEA. Finally, TPF and HT exhibited *in vitro* potent free radical scavenging activity, while they increased (at low concentrations), the levels of GSH, one of the most important antioxidant molecules, in both endothelial cells and myoblasts.

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