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Research Article

Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells

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Olive oil compounds is a dynamic research area because Mediterranean diet has been shown to protect against cardiovascular disease and cancer. Olive leaves, an easily available natural material of low cost, share possibly a similar wealth of health benefiting bioactive phytochemicals. In this work, we investigated the antioxidant potency and antiproliferative activity against cancer and endothelial cells of water and methanol olive leaves extracts and analyzed their content in phytochemicals using LC-MS and LC-UV-SPE-NMR hyphenated techniques. Olive-leaf crude extracts were found to inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary bladder carcinoma (T-24) and bovine brain capillary endothelial (BBCE). The dominant compound of the extracts was oleuropein; phenols and flavonoids were also identified. These phytochemicals demonstrated strong antioxidant potency and inhibited cancer and endothelial cell proliferation at low micromolar concentrations, which is significant considering their high abundance in fruits and vegetables. The antiproliferative activity of crude extracts and phytochemicals against the cell lines used in this study is demonstrated for the first time.

Keywords: Angiogenesis / Cancer / LC-SPE-NMR / Oleuropein / Olive leaf extract

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1 Introduction

Epidemiological studies provide robust evidence for a protective effect of the Mediterranean diet against cardiovascular disease and cancer [1 – 5]. These findings prompted scientists to contemplate Mediterranean flora as a rich source of bioactive phytochemicals with a potential to evolve into preventive and possibly therapeutic agents [6 – 10].

In the era of molecularly targeted, rationally designed cancer therapeutics, natural environment, and especially plants continue to provide a rich source of anticancer agents. Characteristically, 67% of established drugs may be traced to natural origin and approximately half of 155 active small molecules identified from around the 1940s to date are either natural products or directly derived therefrom [11 – 13]. However, the majority of several thousands of plant extracts tested at the National Products Branch of the NCI's Developmental Therapeutics Program during the last two decades originated from Africa, America, and Asia, whereas Mediterranean flora has been poorly represented [14].

Olive tree (*Olea Europaea*) products are essential elements of Mediterranean diet. To date research has mostly focused in olive oil, the key ingredient of Mediterranean diet, and its disease-preventing effects have been attributed to its fatty acid profile, as well as the presence of a number of bioactive components such as tocopherols, phospholipids, and phenolic compounds [15 – 19]. Apart from studies focused in olive oil composition, other studies have consid-

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Abbreviations: BBCE, bovine brain capillary endothelial cell; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl radical; **MCF-7,** human breast adenocarcinoma cell; **OLE,** Olive-Leaf Extract; **T-24,** human urinary bladder carcinoma cell

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ered the constituents present in olive leaf extracts (OLE), mainly because of the availability and low cost of raw material in the region of Mediterranean basin [20, 21]. The antioxidant activity of OLE has been documented and, on this base, this natural product is advertised as an ingredient in complementary alternative medicines and nutritional supplements in western countries [22 – 24]. Recent studies indicate olive-leaf products to contain potent compounds with pleiotropic antiviral and molecular targeted anticancer activities [25 – 29].

The principle components of OLE are shown to be biophenolics, the secoiridoid oleuropein, and their biodegradation products [30]. However, concentrations of active phytochemicals may vary by developmental phase, season of the year, climatic conditions and genetic diversity of tree lineage [31, 32]. Moreover no standardized extraction method has been established for isolation of bioactive constituents from olive leaves and systemic biochemical and molecular investigation of these products has been limited [8].

The aim of this work was to screen extracts from olive leaves for potential biological activity and identify the principal bioactive phytochemicals. Thus, crude olive leaves extracts were investigated for antioxidant potency, screened for antiproliferative activity in endothelial and two cancer cell lines and analyzed using LC-MS and LC-UV-SPE-NMR to identify their major components. The identified compounds were further tested for their antioxidant activity and their ability to inhibit proliferation of cell lines already used for antiproliferative activity of the extracts.

2 Materials and methods

2.1 Plant material, reagents and standards

The olive tree (*O. europaea*) was grown in North west Greece and the olive leaves were collected on November 2005. The leaves were washed, dried in open air, and stored in freeze. ACN, and water were of HPLC grade and were obtained from Scharlau (Barcelona, Spain). Acetic acid and methanol was of analytical grade provided by Merck (Darmstadt, Germany). ACN-d_3 (NMR quality) was purchased from Deutero (Kastellaun, Germany). Oleuropein, hydroxytyrosol, luteolin-7-*O*-glucoside, luteolin-4′-*O*-glucoside, and luteolin were obtained from Extrasynthese (Genay, France). Synthesized hydroxytyrosol acetate was kindly donated by Professor C.Tringali [33]. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$) was purchased from Sigma (St. Louis, MO).

2.2 Sample preparation

Fresh whole leaves (50 g) were boiled with 250 mL distilled water for 1 h and filtered. The water was totally removed with freeze-dryer (CHRIST Alpha 1 – 2, Germany) to obtain the aqueous olive leaf dried extract (AOLE). In order to prepare the methanol olive leafextract (MOLE) 50 g olive leaves were macerated in 250 mL of methanol for 7 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under vacuum.

2.3 Determination of the DPPH$^\bullet$ radical scavenging activity

The scavenging activity was evaluated using the DPPH$^\bullet$ test [34]. 2 mL of each sample were mixed with 1 mL of 0.3 mM solution of DPPH$^\bullet$ in methanol, and the absorbance of the mixture was measured after 30 min incubation time in the dark at 517 nm. Several concentrations of each sample were tested and the% of free radical scavenging activity was determined by the following equation:

$$\% \text{ scavenging activity} = 100 - [(\text{Ab of sample} - \text{Ab of blank}) \times 100/\text{Ab of control}]$$

EC_{50} values are referred to the lower concentration of the compounds under study required for the 50% of the antioxidant reactivity. The measurements were carried out on an UV/Vis spectrophotometer Jenway 6505 (Essex, England).

2.4 Separation and identification of the OLE components

2.4.1 Instrumentation

LC-UV-SPE-NMR measurements were carried out on a chromatographic separation system consisting of an Agilent G13311A solvent delivery pump and a Bruker DAD UV detector (Bruker BioSpin, Rheinstetten, Germany). The samples were injected using an Agilent G1311A autosampler with a 100 µL loop. The Bruker/Spark Prospect 2 SPE unit (Bruker BioSpin and Spark, Emmen, The Netherlands) was used to automatically trap the chromatographic peaks on Hysphere SH cartridges (2 mm id, 15 – 25 µm) after postcolumn addition of water using a Knauer K 120 HPLC pump (Berlin, Germany). The trapped peaks were dried with dry nitrogen gas and eluted with deuterated ACN into a Bruker AV-500 NMR spectrometer equipped with 4 mm LC SEI ^{13}C-^{1}H probe head with an active volume of 60 µL from Bruker BioSpin. The NMR system was controlled by software TopSpin 1.3.

2.4.2 LC-UV-SPE-NMR analysis

The chromatographic separation was carried out on a 250 mm × 4.6 mm id, 5 µm, discovery C18 column from Supelco (Pennsylvania, USA). The flow rate was 0.6 mL/min, and the injection volume was 100 µL. The working solutions of both extracts (20 mg/mL) and standard compounds were dissolved in a mixture of 50% water and 50% ACN. Gradient elution was performed using solvent A (1% acetic acid in water) and solvent B (ACN) with the follow-

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ing linear gradient: at 0 min 5% B, at 20 min 25% B, at 40 min 50% B, at 50 min 80% B, and at 60 min 5% B. During the chromatographic separation, water with 1% acetic acid was added to the eluent leaving the column with a second pump (make up pump), to lower the elution strength and to provide proper retention of the peaks under study on the SPE cartridges (Spark, Holland). When the chromatographic separation was finished, the cartridges were dried with nitrogen gas for 45 min to remove the residual solvents, and subsequently, the analytes were transferred to the NMR probe head with ACN–d_3. The total amount of ACN–d_3 that was used for the elution and transfer of sample was 380 μL for the 60 μL flow probe. All cartridges were automatically reconditioned with 1 mL of ACN at a flow rate of 1 mL/min after use and equilibrated with 1 mL of water at a flow rate of 1 mL/min before each use. Concerning the quantification analysis seven-point calibration curves for the commercially available compounds were used and the regression coefficient values (R) were 0.98 for all compounds. ^{1}H NMR spectra were acquired using a pulse sequence based on the 1-D version of the NOESY sequence, with double presaturation suppressing residual water and ACN signals. The 32 K data points were recorded with a sweep width of 12019 Hz, an acquisition time of 0.1 s, relaxation time 2.4 s, and 256 scans. To process the data acquired, a line broadening of 0.3 Hz was used.

2.4.3 LC-MS measurement

The analysis was performed following the chromatographic conditions referred above. The LC system was equipped with a DAD and mass detector in series (Agilent 1100 series LC/MSD Trap). The system contained an Agilent G1312A binary pump, an Agilent G1313A autosampler, an Agilent G1322A degasser and an Agilent G1315B DAD controlled by Agilent software (Agilent Technologies, Waldbronn, Germany). The mass detector was an Agilent G2455A IT mass spectrometer equipped with an electro-spray ionization (ESI). The scan mass spectra of the phenolic compounds were measured from m/z 50 up to 800.

2.5 Evaluation of antiproliferative activity

2.5.1 Cell cultures

Human endothelial cells from bovine brain (BBCE) were cultured in DMEM medium supplemented with 10% newborn calf serum (NCS), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. MCF-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. T-24 cells (human urinary bladder carcinoma) were cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. All media and sera for cell culture were purchased from Invitrogen and were endotoxin-free.

2.5.2 Proliferation assays

Cell proliferation assays using BBCE cells were previously described [35, 36]. Briefly, cells were plated in 12-well plates at 0.5×10^4/well and the next day received 10 μL of FGF-2 (5 ng/mL) and 5 μL of serial dilutions of extracts from olive leaves or serial dilutions of pure compounds (luteolin 7-*O*-glucoside, oleuropein, hydroxytyrosol, and hydroxytyrosol acetate); all resuspended in DMSO/ethanol, 1:1 by volume. Negative control wells received only 5 μL of DMSO/ethanol, 1:1 volume, whereas positive control cells received the same solvent plus FGF-2, a mitogen of endothelial cells. The same treatment was repeated two days later and the cells were counted with Coulter counter two days thereafter. The same procedure was followed for MCF-7 and T-24 cells except that addition of FGF-2 was omitted.

3 Results and discussion

3.1 Determination of antioxidant capacity and evaluation of antiproliferative activity of the OLEs

Preparations of OLEs were first tested for antioxidant activity using the DPPH$^\bullet$ scavenging assay. A profound antioxidant activity was demonstrated for the majority of OLE preparations. The antioxidant potency *per se* is a well established biomarker, which indicates beneficial biological effects of plant derived phenolic substances [37–39]. We selected the aqueous OLE, AOLE, for subsequent analysis on the criteria of its antioxidant potency (half maximal effective concentration (EC_{50}) = 49 μg/mL), easiness of preparation and water solubility, which indicates water solubility for its active compounds, a very desirable physical property for potential drugs [40, 41]. MOLE was found to scavenge DPPH$^\bullet$ at a concentration of 71 μg/mL and was also further studied. Thus, AOLE and MOLE were tested for cell growth inhibition on two cancer and one endothelial cell lines as *in vitro* assay of putative antimitotic and antiangiogenic potential. It was found that both extracts inhibited cell proliferation of MCF-7, T-24 and BBCE cells at half-maximal concentrations of 209, 178, and 66 μg/mL for AOLE and 174, 510, and 175 μg/mL for MOLE, respectively. This is the first evidence of antiproliferative activity of olive leaves extracts against the cancer cell lines MCF-7, T-24, and the endothelial BBCE cell line. These findings are in line with recently demonstrated cancer inhibitory effects of phytochemical derivatives of olive tree, such as oleuropein, luteolin, and hydroxytyrosol [42, 43]. Moreover, the growth inhibitory effects of the extract on endothelial cells were not of surprise, as we have previously demonstrated that flavonoids exert potent *in vitro* and *in vivo* inhibition of angiogenesis [35, 44].

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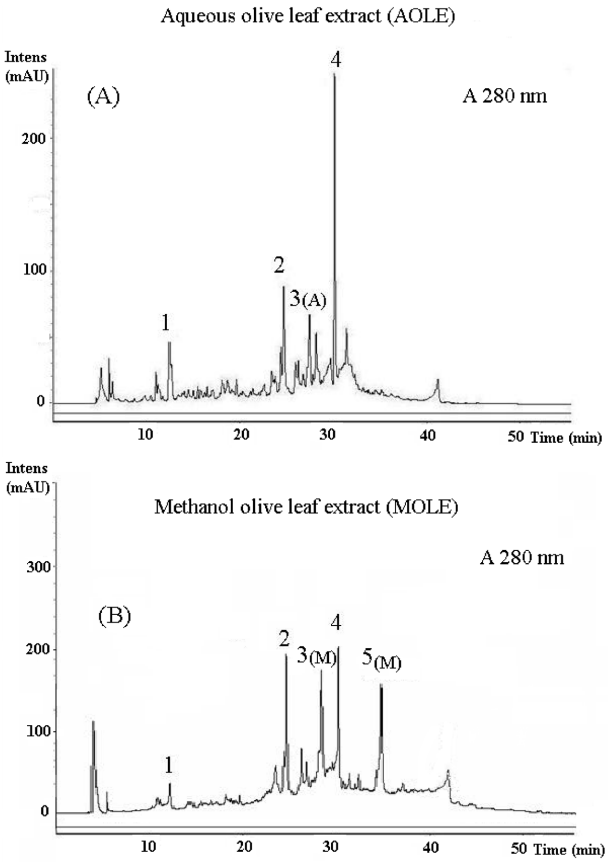

Figure 1. Chromatographic separation of (A) aqueous olive leaf extract (AOLE) and (B) methanol olive leaf extract (MOLE). The chromatograms were recorded at 280 nm.

3.2 Separation and identification of the AOLE and MOLE components

The next step was to separate the extracts and identify the main compounds present. Following the separation conditions described in the experimental section, the LC-UV trace revealed four major peaks for AOLE and five for MOLE sample, as shown in Fig. 1. In a next chromatographic run, the separated peaks were diluted postcolumn with water, they were multiple-trapped onto SPE cartridges and transferred with CD_3CN to the 60 μL LC-NMR probe of the spectrometer in order to acquire their ^{1}H-NMR spectra, as described in previous work of our group [45–47]. The trapping procedure was optimized for the standard oleuropein. In parallel, the extracts were analyzed with LC-MS and the corresponding MS data for each of the peaks of interest were collected. The MS measurements were obtained under ESI in negative mode.

As indicated in Fig. 1, there are three compounds that are present to both AOLE and MOLE samples, denoted with no. 1, 2, and 4. Peak 4, which is eluted at 30 min, is the main component of both extracts. The retention times and UV spectra indicate that this analyte may be attributed to oleuropein. Indeed, the ^{1}H-NMR spectrum of peak 4 confirmed the spin system of the oleuropein moiety. In Fig. 2, in which the ^{1}H-NMR spectrum of peak 4 of AOLE is presented, the vinyl proton H-5 (δ = 7.45 ppm), the characteristic resonance of H-8 appearing at 5.98 ppm as a quartet, and the signal of H-6 at 5.76 ppm as a singlet can easily be distinguished. Moreover, the methyl group of C-9 appears as a doublet at 1.59 ppm, and the aromatic spin system is characteristic of a 1,2,4-trisubstituted benzene. The above assignment is in agreement with the respective NMR spectrum of the standard oleuropein in CD_3CN. The presence of oleuropein was further certified with data of LC-MS (ion mass of 539.4) [30].

The UV spectrum of peak no. 2 consists of two absorption bands at 260–270 and 340–350 nm, which is indicative for flavonoids. Flavonoids and their glycosides can be recognized in a ^{1}H NMR spectrum from their distinctive spin–spin coupling patterns in the aromatic region and the presence of a deshielded signal in the region of 11–13 ppm, which is attributed to the hydroxyl protons OH(5) [48]. The ^{1}H NMR spectrum of peak 2 in CD_3CN indicated a significantly deshielded signal at 13.00 ppm and the characteristic pattern of flavonoid rings. The sugar region of the spectrum revealed one anomeric hydrogen signal at 5.11 ppm (H-1″, d, J = 7.3 Hz). The total ion mass can be of importance for the glycoside part of the molecule, which in combination with the chemical shifts and J-couplings of the anomeric protons of the glycosides suggested the β-configuration for the sugar. The fragmentation pattern of peak no. 2 (total ion at 447 and a daughter ion at 285 m/z) suggested that this analyte is a luteolin glycoside. Comparison of the ^{1}H NMR spectrum of peak no. 2 with that of standard luteolin indicate that peak no. 2 can be attributed to luteolin-7-*O*-glucoside, which is already been reported as an OLE flavonoid [21]. The result was confirmed from the identical NMR and MS spectra of the standard compound and spiking procedure. Accordingly, peaks no $3_{(M)}$ and $5_{(M)}$ were ascribed to luteolin-4′-*O*-glucoside and luteolin, respectively.

Peak no. 1 and $3_{(A)}$ showed similar UV and ^{1}H-NMR spectra. The chemical shifts and coupling constant pattern of the signals in the aromatic region indicated the presence of hydroxytyrosol moiety in both cases (Fig. 3). In the ^{1}H-NMR spectrum of peak $3_{(A)}$, the signal at 4.19 ppm can be attributed to H-1 protons. Taking into consideration the respective total ion mass data at 153 and 195 m/z, peaks no. 1 and 3 can be attributed to hydroxytyrosol and hydroxytyrosol acetate, respectively. This can also explain the high frequency shift for the side-chain aliphatic proton in comparison to that of free hydroxytyrosol. It is worth noticing that although both compounds have been identified in olive oil [49], hydroxytyrosol acetate has only recently been reported to be present in olive leaves extract [50]. According to Gordon *et al*., hydroxytyrosol acetate possesses antioxidant activity similar to that of hydroxytyrosol [51].

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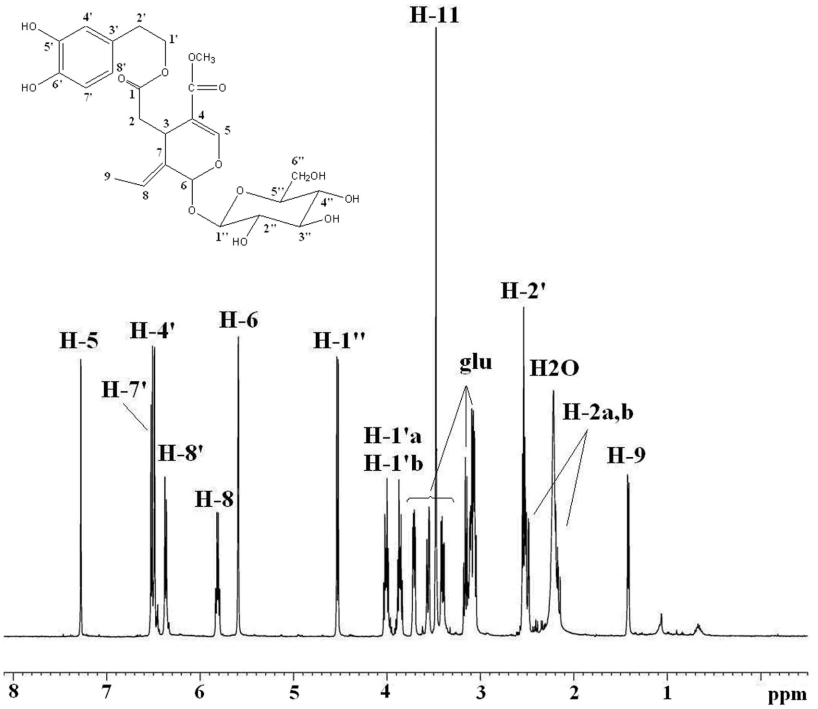

Figure 2. 500 MHz ^{1}H NMR spectrum of peak 4. The signals are attributed to the secoiridoid oleuropein.

The spectral data and retention times of each analyte are summarized below. Compound 1 (hydroxytyrosol), t_R = 12.56 min, *m/z* 153 [M$^-$]. ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 6.75 (1H, d, J = 8.1 Hz, H-7); 6.72 (1H, *d*, J = 1.8 Hz, H-4); 6.60 (1H, dd, J_1 = 8.1 and J_2 = 1.8 Hz, H-8); 3.66 (2H, t, H-1); 2.67 (2H, t, H-2).

Compound 2 (luteolin-7-*O*-glucoside), t_R = 24.75 min, *m/z* 447 [M$^-$], MS^2 *m/z* 285 [M-$C_6H_{10}O_5$]$^-$. ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 13.01 (1H, s, OH-5); 7.44 (1H, s H-2′); 7.42 (1H, d, J = 8.3 Hz, H-6′); 6.95 (1H, d, J = 8.3 Hz, H-5′); 6.61 (1H, s, H-3); 6.43 (1H, s, H-8); 6.26 (1H, s, H-6); 5.11 (1H, d, J = 7.3 Hz, anomeric proton H-1″); 3.00–4.00 (sugar protons).

Compound 3$_{(A)}$ (hydroxytyrosol acetate), t_R = 27.52 min, *m/z* 195 [M$^-$]. ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 6.75 (1H, d, J = 8.0 Hz, H-7); 6.73 (1H, d, J = 1.8 Hz. H-4); 6.62 (1H, dd, J_1 = 8.0 and J_2 = 1.8 Hz, H-8); 4.19 (2H, *t*, H-1); 2.78 (2H, *t*, H-2); H-10 signal is overlapped by ACN–H_2O and therefore, it is eliminated.

Compound 3$_{(M)}$ (luteolin-4′-*O*-glucoside), t_R = 28.04 min, *m/z* 447 [M$^-$], MS^2 *m/z* 285 [M-$C_6H_{10}O_5$]$^-$. ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 12.77 (1H, s, OH-5); 7.42 (1H, s, H-2′); 7.39 (1H, d, J = 8.9 Hz, H-6′); 7.07 (1H, d, J = 8.6 Hz, H-5′); 6.55 (1H, s, H-3); 6.43 (1H, s, H-8); 6.15 (1H, s, H-6); 5.97 (1H, d, J = 6.60 Hz, anomeric proton H-1″); 3.00–4.00 (sugar protons).

Compound 4 (oleuropein), t_R = 30.06 min, *m/z* 539 [M$^-$], ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 7.45 (1H, s, H-5); 6.69 (1H, d, J = 8.0 Hz, H-7′); 6.66 (1H, d, J = 1.9 Hz, H-4′); 6.54 (1H, dd, J_1 = 8.0 and J_2 = 1.9 Hz, H-8′); 5.98 (1H, q, H-8); 5.76 (1H, s, H-6); 4.53 (1H, d, J = 7.9 Hz, Hanomeric proton H-1″); 4.18 (1H, m, H-1′a); 4.04 (H1, m, H-1′b); 3.65 (3H, s, H-11); 3.88–2.00 (sugar protons); 2.71 (2H, m, H-2′); 2.66 (1H, d, J = 4.0 Hz, H-2a); 2.37 (1H, d, J = 4.0 Hz, H-2b); 1.59 (3H, d, H-9).

Compound 5$_{(M)}$ (luteolin), t_R = 34.60 min, *m/z* 285.0[M$^-$], ^{1}H-NMR (500 MHz, ACN–d_3) δ (ppm) = 12.72 (1H, s, OH-5); 7.24 (1H, s, H-2′); 7.22 (1H, d, J = 8.3 Hz, H-6′); 7.17 (1H, d, J = 8.6 Hz, H-5′); 6.37 (1H, s, H-3); 6.30 (1H, s, H-8); 6.03 (1H, s, H-6).

In addition, the concentration levels of the identified compounds were chromatographically determined by applying seven-point calibration curves for the commercially available compounds. Quantification analysis showed that oleuropein is the major compound present with a concentration level up to 35% of the AOLE and up to 10% of the MOLE as indicated in Table 1. The regression coefficient values (R) were 0.98 for all compounds studied.

3.3 Determination of antioxidant capacity and evaluation of antiproliferative activity of the identified phytochemicals and of the artificial mixtures of them

In our attempt to correlate the antioxidant activity of the extracts and their phytochemical content, the antioxidant activity of each of the identified compounds was tested. Commercially obtained luteolin, luteolin 4′-*O*-glucoside, luteolin 7-*O*-glucoside, oleuropein, hydroxytyrosol, and hydroxytyrosol acetate (kindly donated by Professor C. Tringali), were all tested to define their free radical scavenging activity. The EC_{50} values are presented in Table 1;

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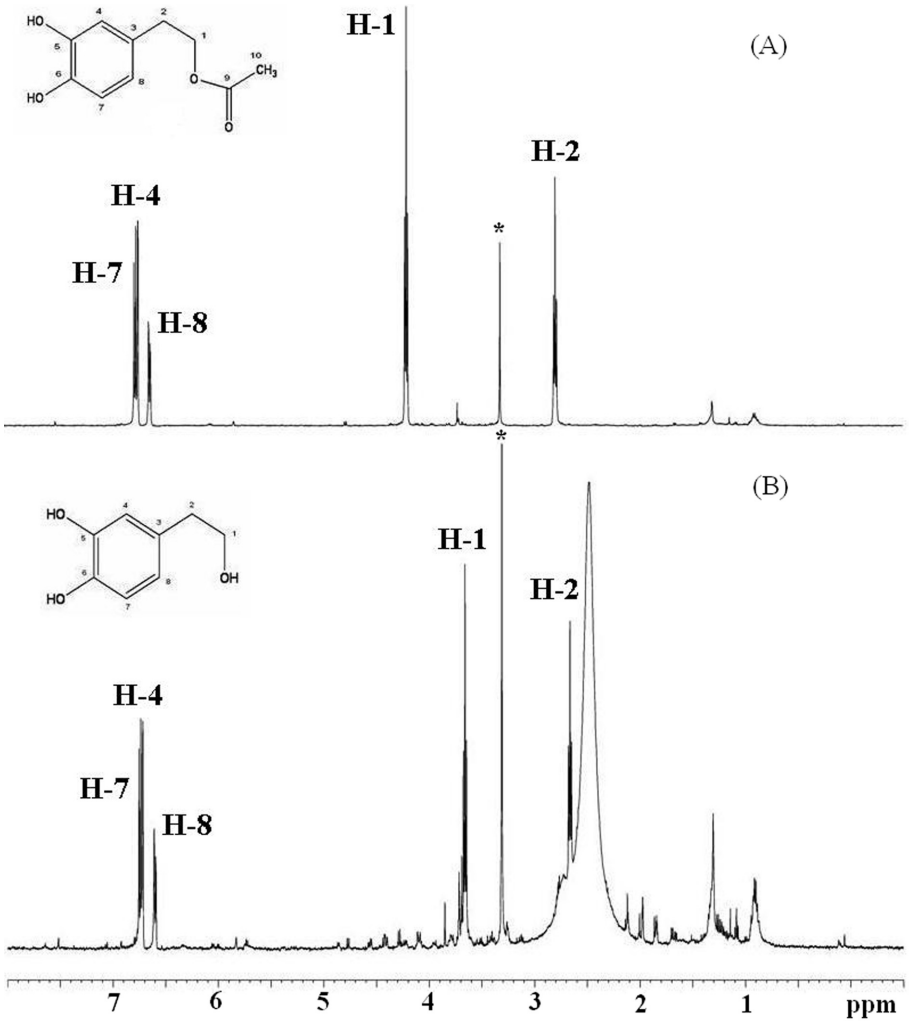

Figure 3. 500 MHz ^{1}H NMR spectra of (A) the trapped peak 1, hydroxytyrosol and (B) the trapped peak $3_{(A)}$, hydroxytyrosol acetate (the H-10 signal is overlapped by the CD_3CN-H_2O residual signals and, therefore, it is eliminated.)

Table 1. Concentration levels of the major compounds present in AOLE and MOLE samples together with the respective antioxidant activity (EC_{50}) and inhibitory effect (IC_{50}) against cancer and endothelial cell proliferation data

Sample	Concentration levels in AOLEmg/g extract[a)]	Concentration levels in MOLEmg/g extract[a)]	EC_{50} (μM)	IC_{50}[b)] (μM)		
				BBCE	T24	MCF7
Hydroxytyrosol	20.1 ± 1.3	18.3 ± 0.1	35.84	2.44 ± 1.08	12.39 ± 2.20	24.86 ± 8.15
Luteolin-7-*O*-glucoside	90.6 ± 2.2	67.8 ± 1.5	23.19	3.91 ± 0.88	11.35 ± 0.32	40.85 ± 5.01
Luteolin-4′-*O*-glucoside	ND[c)]	70.6 ± 0.5	32.84	30.86 ± 1.36	31.69 ± 2.52	10.83 ± 0.29
Oleuropein	345.3 ± 5.8	92.5 ± 1.6	34.56	2.61 ± 0.09	7.59 ± 1.80	12.00 ± 0.62
Luteolin	ND	53.5 ± 0.4	29.37	9.69 ± 0.69	28.17 ± 4.6	9.07 ± 0.53
Hydroxytyrosol acetate	17.7 ± 1.1	ND[c)]	41.99	9.34 ± 1.61	23.32 ± 0.38	28.67 ± 8.10

a) Mean value of two measurements.
b) Mean value of three measurements.
c) Not detected.

luteolin and its glucosides are the most active compounds, followed by oleuropein, hydroxytyrosol, and its acetate derivative. The same standards were used to determine their half-maximal inhibitory concentrations on the cancer cell lines MCF-7 and T-24 and the endothelial cell line BBCE, as shown in Fig. 4. All compounds exhibited inhibitory effects on cancer and endothelial cell proliferation (Table 1). We have not observed any difference in the percentage of nonviable BBCE, T-24, and MCF-7 cells in the test wells *versus* the control wells, which have received only the solvent (DMSO/ethanol. 1/1 by volume), indicating that there was no appreciable cytotoxicity (trypan blue assay; data not

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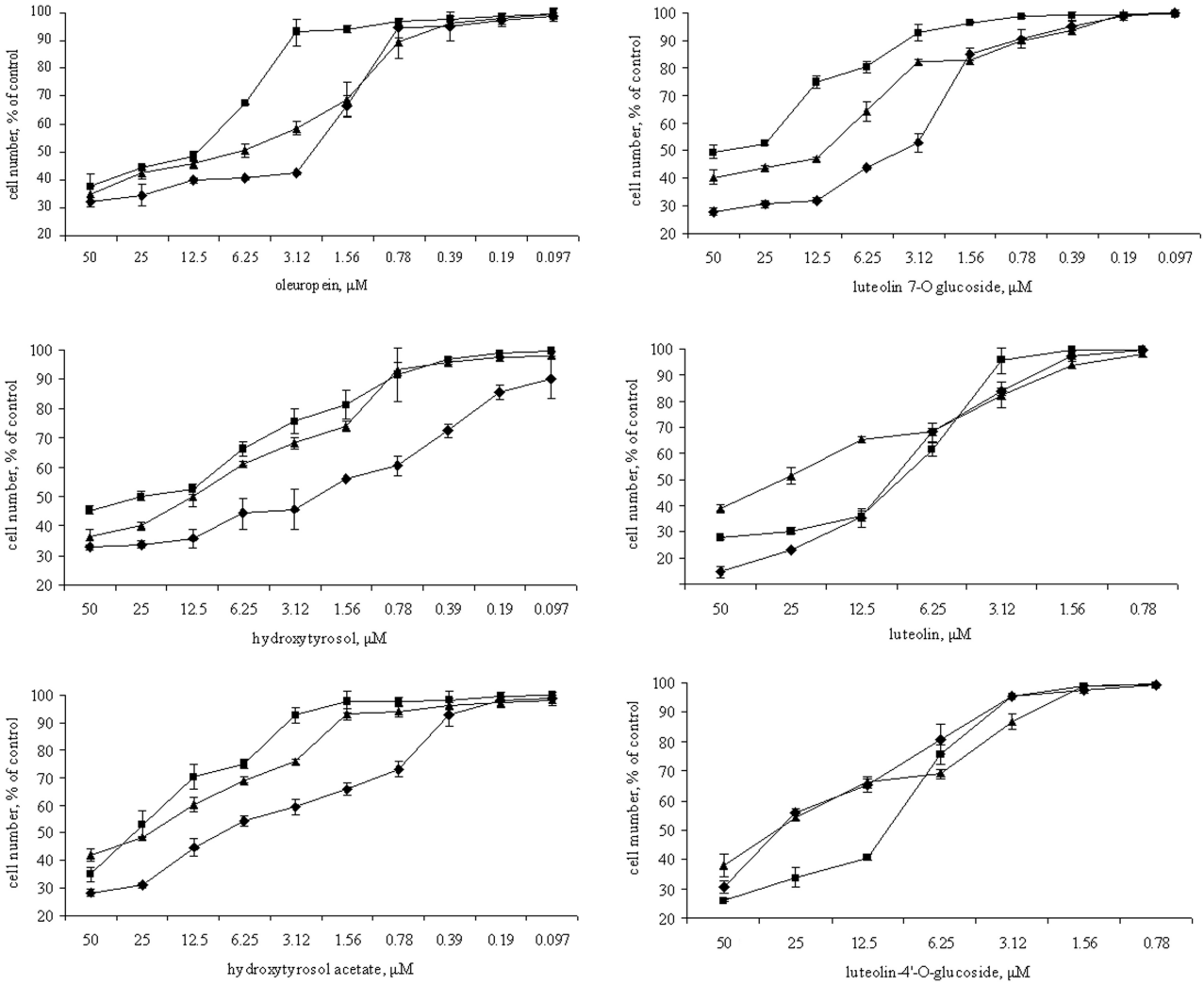

Figure 4. Effect of different concentrations of oleuropein, luteolin-7-*O*-glucoside luteolin-4′-*O*-glucoside, luteolin, hydroxytyrosol and its acetate derivative on the proliferation of human breast adenocarcinoma (–■– MCF-7), human urinary bladder carcinoma (–▲– T-24) and bovine brain capillary endothelial (–♦– BBCE) cells.

shown). It is interesting to note that the half-maximal inhibitory concentrations of luteolin 7-*O*-glucoside, oleuropein, hydroxytyrosol, and hydroxytyrosol acetate for proliferating endothelial cells (IC_{50} 2.4 to 9.3 μM), compared favorably with that of the nonconjugated luteolin, a compound, for which we have shown that exhibits *in vitro* and *in vivo* antiangiogenic activity by inhibiting vascular endothelial growth factor (VEGF)-induced signaling in endothelial cells (IC_{50} 5 μM) [44, 52]. It should be noted that though oleuropein has been suggested to possess pharmacological properties since the early 70's, its anticancer effects have only recently started being investigated [25, 28]. More data exist regarding the anticancer properties of hydroxytyrosol and luteolin [44, 53, 54].

To further investigate these findings and evaluate the extract, artificial mixtures composed of the isolated components in relative amounts (as indicated in Table 1) similar to that of the AOLE and MOLE were prepared and tested for antiproliferative activity against the same cancer and endothelial cells. It was found that the aqueous and methanol artificial mixtures inhibited cell proliferation of MCF-7, T-24, and BBCE cells at IC_{50s} of 72, 100, and 62 μg/mL and 565, 135, and 42 μg/mL, respectively. This data indicate, that the antiproliferative activity of the extracts should mainly be attributed to the identified phytochemicals.

4 Concluding remarks

In summary, this study provides for the first time convincing integrated evidence that OLEs have health protecting biological activity as antioxidants and as potent inhibitors

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of cancer and endothelial cells proliferation. The major components identified were oleuropein, hydroxytyrosol, hydroxytyrosol acetate, and the flavonoids luteolin, luteolin-7-*O*-glucoside, and luteolin-4′-*O*-glucoside. It is demonstrated that those phytochemicals inhibited the proliferation of cancer and endothelial cells with IC_{50} at the low micromolar range. It is worth noticing that OLEs are commercially available as nutritional supplements and their components are commonly found and easily consumed. Advancing research of OLE phytochemicals to a structure-function molecular level and investigation of possible synergism is warranted.

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The authors have declared no conflict of interest.

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