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Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity

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ABSTRACT

Virgin olive oil (VOO) has nutritional and sensory characteristics that make it unique and a basic component of the Mediterranean diet. Its importance is mainly attributed to its richness in polyphenols, which act as natural antioxidants and may contribute to the prevention of several human diseases. In this paper we report the determination and quantification of oleocanthal, one of the main substances responsible for the bitter taste of olive oil, together with a quali-quantitative analysis by HPLC analytical methods of phenolics from Cilento VOO and olive oil pomace. The total phenolic content was also determined and the in vitro antioxidant and free-radical scavenging activities by DPPH test was evaluated. A superoxide anion enzymatic assay was also carried out and the results were confirmed by the inhibition of xanthine oxidase activity assay. The possible protective role played by VOO secoiridoids on injurious effects of reactive oxygen metabolites on the intestinal epithelium, using Caco-2 human cell line, was investigated.

1. Introduction

Cilento National Park (Campania region, Italy) is one of the largest parks in Italy where "Cilento" virgin olive oil (VOO) is obtained from the fruit of several cultivars of olive tree (*Olea europaea* L.). The origin of this oil is guaranteed: this product is defined as a "Protected Designation of Origin" (PDO; EC, 1998) and presents some characteristics of quality and of originality that are the result of the geographical influences and the human factor. Because of its nutritional and biological characteristics, VOO is one of the most important components of the Mediterranean diet and local agriculture (Ferro-Luzzi & Sette, 1989). The traditional Mediterranean diet, which consists of fruits, vegetables, cereals, legumes and fish, is thought to represent a healthy lifestyle; especially the incidence of several cancers (Owen et al., 2004), including colorectal cancer, is lower in Mediterranean countries compared to Northern Europe. Olives and olive derived are an important part of this diet and are recognized as a valuable source of natural phenolic antioxidants (Briante, Febbraio, & Nucci, 2003). In fact, an increasing number of epidemiologic and experimental studies report that the olive oil may have a role in the prevention of coronary heart disease (Stark & Madar, 2002), cognitive impairment, e.g., Alzheimer's disease (Scarmeas, Stern, Tang, Mayeux, & Luchsinger, 2006), protective effects against of the cancer of the colon, breast and ovary (Braga et al., 1998), diabetes accompanied by hypertryglyceridemia and inflammatory and autoimmune diseases, such as rheumatoid arthritis (Alarcón de la Lastra, Barranco, Motilva, & Herrerías, 2001). Also, olive oil has been shown to reduce low-density lipoprotein (LDL) oxidisability in the post prandial state (Hargrove, Etherton, Pearson, Harrison, & Kris-Etherton, 2001). These beneficial health effects of olive oil are ascribable to monounsaturated, and low unsaturated fatty acids and a number of phenolic compounds, usually grouped under the rubric "polyphenols". Phenolic compounds in VOO are a complex mixture of components, that include α- and γ-tocopherols, hydroxytyrosol, tyrosol, phenolic acids (caffeic acid, vanillic acid, syringic acid), lignans (pinoresinol, 1-acetoxypinoresinol) (Montedoro, Baldioli, & Miniati, 1992), and secoiridoids (oleuropein aglycone, oleuropein, demethyloleuropein, ligstroside) (Lavelli & Bondesan, 2005). In recent years it was reported that one of the well-known phenolic compounds present in newly-pressed extra VOO, the dialdehydic form of deacetoxy-ligstroside aglycone, called oleocanthal, is one of the main substances responsible for the bitter taste of olive oil and possesses ibuprofen-like cyclooxygenases (COX-1 and 2) inhibitory activity being so responsible of its anti-inflammatory effect (Beauchamp, Keast, Morel, & Lin, 2005). The content of phenolic compounds is an important parameter in the evaluation of VOO quality because

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phenols largely contribute to oil flavour and aroma and protect the free fatty acid fraction from oxidation (Servili & Montedoro, 2002). The recognized nutritional value of extra VOO is a direct expression of its antioxidant power, namely its ability to inhibit oxidative reactions that are involved in the beginning and progression of many human diseases. The antioxidant capacities of oleuropein, its aglycone, and minor phenols have been studied using different methods such as the DPPH and ABTS tests (Samaniego Sànchez et al., 2007), but very few studies have been made on the antioxidant activities of the leaves and olive oil pomace.

To the best of our knowledge, nothing has been published about "Protected Designation of Origin" VOO and olive pomace produced in "Cilento". In this paper we report the determination and quantification of oleocanthal, together with a quali-quantitative analysis by HPLC analytical methods of phenolics from Cilento VOO and olive oil pomace. The total phenolic content was also determined and in vitro antioxidant and free-radical scavenging activities by DPPH test was evaluated. The antioxidant activity of VOO secoiridoids by radical scavenging activity test, superoxide anion enzymatic generation assay (Cos et al., 1998; Robak & Griglewski, 1988) and xanthine oxidase (XOD) activity assay (Robak & Griglewski, 1988), was also reported. Finally, the possible protective role played by VOO secoiridoids on injurious effects of reactive oxygen metabolites (ROM) on the intestinal epithelium, using Caco-2 human cell line (Baker & Baker, 1993) was investigated.

2. Materials and methods

2.1. Samples

The olive oil samples, the olive pomace, and the leaves of *O. europaea* L. were acquired from "National Park of Cilento" (Campania region, Italy) in olive groves located in the area of Perdifumo (Salerno, Italy) ("La Pepa"), and in the area of Acquamela di Casalvelino (Salerno, Italy) ("Severini").

2.2. Chemicals

Xanthine, xanthine oxidase (XOD), sodium carbonate, sodium phosphate monobasic and sodium phosphate dibasic, neutral red, L-glutamine, and hydrogen peroxide were obtained by Sigma Aldrich (Gillingam, Dorset, UK). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT); penicillin–streptomycin, from porcine pancreas and PBS tablets were purchased from ICN-Flow (Costa Mesa, CA). The solvents were obtained by Carlo Erba Reagents. Nanopure water was prepared by Milli-Q apparatus.

2.3. General methods

HPLC analyses were conducted on an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system G-1312, degasser G-1322A, G-1315A Photodiode Array Detector, G-1328A Rheodyne injection system, and equipped with a Waters C_{18} μ-Bondapak column (3.9 × 300 mm, 10 μm, Waters, Milford, MA, USA). HPLC fractionations were conducted on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column and U6K injector. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); and reagent grade chemicals (Carlo Erba, Milano, Italy) were used throughout. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and Time Proportional Phase Increment (TPPI) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. ESI-MS (positive mode) were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer (San Jose, CA, USA) equipped with Xcalibur software.

2.4. Extraction and HPLC analysis of phenolic compounds from VOO

Phenolic compounds were extracted from VOO according to Montedoro et al. with minor modifications: 8 ml of methanol were added to 8 g of VOO; the mixture was submitted to a vortex for 30 s and the two phases were separated by centrifugation at 3000 rpm for 10 min. The extraction was repeated twice. Alcoholic extracts were then combined and concentrated in vacuum at $T < 35$ °C until a syrupy consistency was reached. Eight millilitre of acetonitrile were added to the extract, and it was partitioned with 8 ml of *n*-hexane. The apolar phases were also purified with 5 ml of acetonitrile. The two phases were then separated by centrifugation at 3000 rpm for 10 min. Finally, the acetonitrile phases were evaporated under a stream of nitrogen to give phenolic fraction residue (200 mg). The phenolic fractions of the VOO were dissolved in acetonitrile and analyzed by RP-HPLC DAD on a C_{18} μ-Bondapak column (3.9 × 300 mm, 10 μm, Waters, Milford, MA, USA, flow rate 0.8 ml min^{-1}). The wavelengths were set at 240, 278, and 320 nm. Chromatograms were acquired at 278 nm. The injection volume was 50 μl (1 mg/50 μl). The solvent gradient changed according to the following conditions (solvent A, H_2O + TFA 0.1% and solvent B, acetonitrile + TFA 0.1%): 0 min, 100% A, 2 min, 95% A; 10 min, 75% A; 10–20 min, 60% A, 20–30 min, 50% A; 30–40 min 100% B. The identification of phenolic compounds was made by the co-injection of correspondent commercial standard of gallic acid, hydroxytyrosol, caffeic acid, tyrosol, and ferulic acid (Extrasynthese, Genay Cedex, France) or pure compounds obtained from the leaves. Phenolic compounds were quantified by standard calibration curve using commercial reference compounds oleuropein and caffeic acid (Extrasynthese, Genay Cedex, France) injected in the same experimental condition used for the analysis.

2.5. Extraction, isolation and identification of phenolic compounds from the leaves

The air-dried powdered leaves of *O. europaea* (500 g) were defatted with petroleum ether and successively extracted for 48 h with $CHCl_3$, $CHCl_3$–MeOH (9:1), and MeOH, by exhaustive maceration (3 × 2 l), to give 14.0, 12.5, 9.0, and 30.0 g of the respective residues. Part of the methanol extract (2.5 g) was chromatographed on Sephadex LH-20 column, using MeOH as eluent. A total of 99 fractions were collected (8 ml each) and combined by TLC results on silica 60 F_{254} gel-coated glass sheets with *n*-BuOH–AcOH–H_2O (60:15:25) and $CHCl_3$–MeOH–H_2O (40:9:1), to give 11 pooled fractions (*A*–*K*). Fraction *C* (110 mg) was purified by RP-HPLC using a C_{18} μ-Bondapak column (7.8 × 300 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (35:65) as eluent to yield oleuropein (t_R = 22.0 min, 18.0 mg), ligstroside aglycone (t_R = 32.0 min, 14.0 mg), and oleuropein aglycone (t_R = 35.0 min, 10.0 mg). Fraction *D* (120 mg) was separated by HPLC DAD on a C_{18} μ-Bondapak column (3.9 × 300 mm, flow rate 1.0 ml min^{-1}) with MeOH–H_2O (45:55) to give gallic acid (t_R = 4.5 min, 1.8 mg), hydroxytyrosol (t_R = 6.3 min, 10.0 mg), caffeic acid (t_R = 7.6 min, 6.0 mg), tyrosol (t_R = 9.1 min, 35.0 mg), and ferulic acid (t_R = 9.6 min, 3.0 mg). Fraction *E* (80 mg) was fractionated by RP-HPLC on C_{18} μ-Bondapak column (7.8 × 300 mm, flow rate 2.5 ml min^{-1}) using MeOH–H_2O

(3:7) as mobile phase to isolate secologanoside 7-methyl ester and pinoresinol. The characterisation of pure compounds was obtained by co-injection with commercial standard (Extrasynthese, Genay Cedex, France) of gallic acid, hydroxytyrosol, caffeic acid, tyrosol, and ferulic acid, and by NMR, ESI-MS analyses, and comparison with the previously published data for ligstroside aglycone (He et al., 2001), oleuropein (Kuwajima, Uemura, Takaishi, Inoue, & Inouye, 1988), secologanoside 7-methyl ester (Machida, Unagami, Ojima, & Kikuchi, 2003), and pinoresinol (Owen et al., 2000).

2.6. Extraction, isolation, and HPLC analysis of phenolic compounds from olive oil pomace

The olive oil pomace (500 g) was defatted with petroleum ether and successively extracted for 48 h with $CHCl_3$, $CHCl_3$–MeOH (9:1), and MeOH, by exhaustive maceration (3 × 2 l), to give 15.0, 20.0, 25.0, and 45.0 g of the respective residues. The methanol extract was chromatographed on Sephadex LH-20 column, using MeOH as eluent, to obtain 70 fractions of 10 ml combined together in six groups. Group 2 was submitted to HPLC DAD on a C_{18} μ-Bondapak column (3.9 × 300 mm, flow rate 0.8 ml min^{-1}) with the following gradient: (solvent A, H_2O, solvent B, MeOH): 0 min, 100% A, 2 min, 95% A, 8 min 75% A, 10 min 60% A, and 10–30 min 100% B. The wavelength was set at 278 nm. The identification of all phenolic compounds has been possible by the co-injection of the correspondent commercial standard (Extrasynthese, Genay Cedex, France) or pure compounds obtained from the leaves.

2.7. Quantification

The quantification of phenolic compounds was carried out using the same HPLC–DAD method applied for the analysis, with the respective standard. To asses the validity of the method, all test parameters were carefully chosen to cover the range of samples and concentrations involved. The linearity of standard curve was expressed in terms of the determination coefficient plots of the integrated peaks area versus concentration of the same standard, and expressed as recovery (%) of phenols. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. The system was linear in all cases ($r > 0.99$). Three replicates on the same day were carried out.

2.8. Extraction and HPLC quantitative analysis of oleocanthal

Oleocanthal was extracted from VOO by liquid–liquid partitioning according to Impellizzeri and Lin (2006), with minor modifications. The residue of methanol/water phase was submitted to HPLC analysis. An Agilent 1100 series HPLC system with UV-DAD detector set to 278 nm was used. Separation was performed on a C_{18} μ-Bondapak column (3.9 × 300 mm, flow rate 0.8 ml min^{-1}). The wavelength was set at 278 nm. The injection volume was 50 μl (1 mg/50 μl). The solvent gradient changed according to the following conditions (solvent A, H_2O + TFA 0.1% and solvent B, acetonitrile + TFA 0.1%): from 0 to 35 min 25% B; from 35 to 45 min 80% B; from 45 to 55 min, 100% B. The chromatogram showed the presence of oleocanthal (t_R = 25.4 min). The identification of this compound was possible by comparing its physical properties with those reported in literature (Impellizzeri & Lin, 2006) and by ESI-MS data (m/z 304.13). Since oleocanthal does not seem to be stable for a long period of time, another stable compound for internal calibration was used. After several screening experiments, gallic acid was chosen as internal standard for its UV absorbance at 278 nm and chromatographic retention time.

2.9. Quantitative determination of total phenols

The polar extracts of VOO, pomace, and leaves were dissolved in MeOH and analyzed for their total phenolic content according to the Folin–Ciocalteu colorimetric method (Di Stefano & Guidoni, 1989; Picerno, Mencherini, Lauro, Barbato, & Aquino, 2003). Total phenols were expressed as oleuropein equivalents (mg/kg).

2.10. Bleaching of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH test)

The antioxidant activities of VOO, pomace, and leaves polar extracts and positive control (α-tocopherol and oleuropein) were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) with the procedures described by Rapisarda et al. (1999). DPPH has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 μl) of the MeOH solution containing different amounts of the VOO, pomace, and leaves polar extracts and control was added to 1.5 ml of freshly prepared DPPH solution (0.025 g/l in MeOH); the maximum concentration employed was 300 μg/ml. An equal volume (37.5 μl) of MeOH was added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV–visible spectrophotometer 10 min after starting the reaction. The DPPH concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH (%$DPPH_{REM}$) was calculated as

$$\%DPPH_{REM} = [DPPH]_T/[DPPH]_0 \times 100$$

where T is the experimental duration time (10 min). All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated by using the Litchfield and Wilcoxon test (Tallarida & Murray, 1984).

2.11. Superoxide anion enzymatic generation assay

Superoxide anion was generated in an enzymatic system by preparing a mixture of xanthine and xanthine oxidase (XOD). The reaction mixture included 0.1 mM EDTA, 50 μg/ml bovine serum albumine (BSA), 25 μM nitroblue tetrazolium, 0.1 mM xanthine and 3.3×10^{-3} units XOD in 40 mM sodium carbonate buffer (pH 10.2) in a final volume of 3 ml. After incubation at 25 °C with increasing concentrations of samples, the absorbance of formazan produced was determined at 560 nm. The inhibitory effect of samples on the generation of superoxide anion were estimated by the equation: inhibitory ratio = $(A_0 - A_1) \times 100/A_0$; where A_0 is absorbance with no addition of sample and A_1 is absorbance with addition of sample. Inhibitory ratio for each sample was plotted as a function of the concentration; then the IC_{50} value, with the statistical method of linear regression was calculated.

2.12. Xanthine oxidase inhibition assay

XOD inhibition activity was evaluated by the spectrophotometric measurement of the formation of uric acid by xanthine. A 100 μM solution of xanthine in 0.1 M phosphate buffer pH 7.8 with 0.04 units/ml of XOD was incubated for 10 min at room temperature and read at 295 nm against a blank sample. Various concentrations of testing compounds were added to samples before the enzyme has been instilled and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} values.

2.13. Cell cultures

Caco-2 cells were routinely maintained in DMEM, containing 200 ml/l FCS, 10 ml/l of 100× nonessential amino-acids,

2 mmol/l L-glutamine, 5×10^4 IU/l penicillin, 50 mg/l streptomycin at 37 °C in a 5% CO_2 atmosphere at 90–100% relative humidity. Cell were grown in 10 cm Petri dishes. For experiments, cells were seeded at a density of 90,000 cells/cm^2 in a Transwell insert, and the medium (0.1 ml in the insert and 0.8 ml in the well) was changed every 48 h. Fourteen to sixteen days after confluence, the integrity of the monolayer of differentiated cells was monitored according to the method of Hildago, Raub, and Borchardt (1989).

2.14. Induction of oxidative stress

An iron-free medium (EMEM) was used for the oxidative stress induction experiments. The oxidative stress was induced in the apical compartment of the transwell insert by two methods: (1) addition of H_2O_2 and (2) an enzymatic system, composed of different amounts of XOD and its substrate xanthine (250 μmol/l). After 20 h of incubation, several oxidative stress markers were measured. To assay the capacity of secoiridoids to protect Caco-2 cells from ROM-mediated oxidative injury, cells were preincubated for 4 h with compounds, which had been added to the apical side of the monolayer. At the end of the preincubation time, the medium was changed before the addition of the oxidative stress-inducing agents.

2.15. Neutral red assay

We assessed the cytotoxicity of ROM on Caco-2 by the viability test of neutral red uptake, performed according to the procedure of Fautz, Husein, and Hechenberger (1991). After oxidative stress induction the medium in the insert was removed and replaced with 0.1 ml of fresh medium containing 1.14 mmol/l neutral red. At the end of the 3 h incubation, the medium was removed and cells were washed twice with PBS; finally the incorporated neutral red was released from cells by incubation for 15 min at room temperature in the presence of 1 ml of cell lysis buffer containing acetic acid (1% v/v) and ethanol (50% v/v). To measure the dye taken up, the cell lysis products were centrifuged and supernatants spectrophotometrically measured at 540 nm.

3. Results and discussions

3.1. Quantitative analysis of oleocanthal

A simple reverse phase HPLC method using UV detection at 278 nm, was carried out to quantitatively determine natural anti-inflammatory oleocanthal in Cilento VOO extracts (Impellizzeri & Lin, 2006; Smith, Han, Breslin, & Beuchamp, 2005). The result showed that the total content of oleochantal, expressed as gallic acid equivalent, was 5%, an amount quite comparable with that reported for VOO coming from other geographical areas (Franconi et al., 2006).

3.2. Phenolic composition

The analysis of phenolic substances using reversed phase-HPLC from VOO and olive oil pomace "La Pepa" and "Severini", as described in the experimental section, allowed to the separation and identification of several phenolic compounds. As shown in Fig. 1 and Table 1, phenolic compounds identified and quantified in the two VOO belong to two classes: simple phenols (gallic acid t_R = 6.4 min, hydroxytyrosol t_R = 7.7 min, tyrosol t_R = 12.0 min, caffeic acid t_R = 13.4 min, syringic acid t_R = 16.3 min, and ferulic acid t_R = 21.2 min) and secoiridoid derivatives (oleuropein t_R = 16.9 min, ligstroside aglycone t_R = 17.9 min, and oleuropein aglycone t_R = 19.8 min). A chromatogram of olive oil pomace (data not shown) showed the presence of gallic acid, hydroxytyrosol, tyrosol, caffeic acid, oleuropein, ligstroside aglycone, oleuropein aglycone, ferulic acid, and vanillic acid. The amount of phenolic compounds is an important factor when evaluating the quality of VOO because of their involvement in its resistance to oxidation and its sharp bitter taste (Morello, Motilva, Tovar, & Romero, 2004) and for the antioxidant properties attributed to VOO from the recent scientific literature (Bendini et al., 2007; Samaniego Sànchez et al., 2007). From both a qualitative and quantitative point of view the two VOO and olive oil pomace are quite similar. In all the samples studied, secoiridoids comprised about 50–70% of the total phenolic derivatives, with oleuropein and ligstroside aglycone being the most abundant compounds. Among simple phenols, gallic acid, hydroxytyrosol, and tyrosol are the major constituents in VOO,

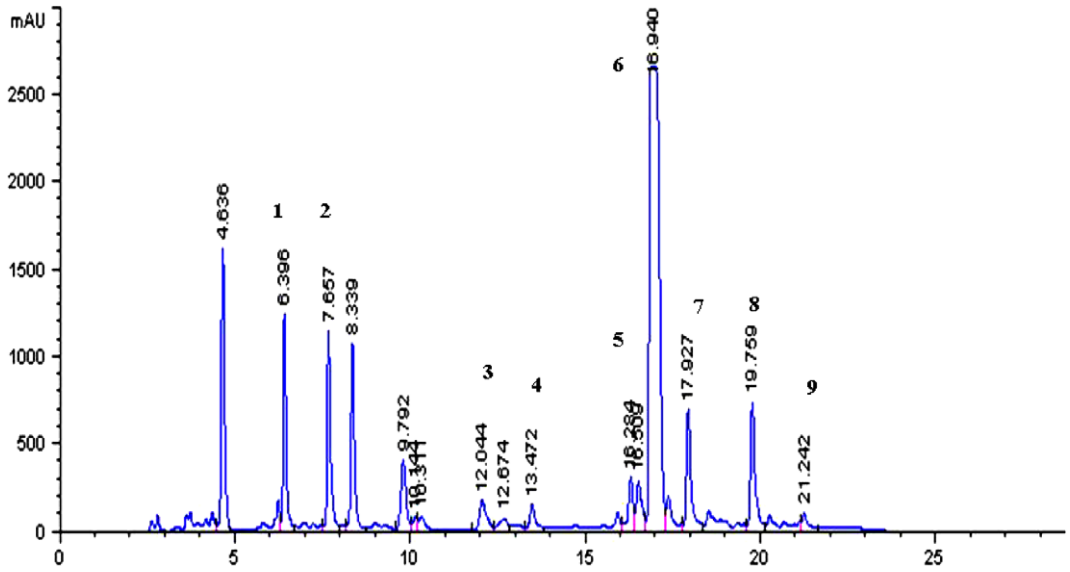

Fig. 1. HPLC–DAD chromatogram of "La Pepa" VOO acquired at 278 nm. Identified compounds: (1) gallic acid; (2) hydroxytyrosol; (3) tyrosol; (4) caffeic acid; (5) syringic acid; (6) oleuropein; (7) ligstroside aglycone; (8) oleuropein aglycone; (9) ferulic acid.

Table 1
Phenol composition (mg/kg) in Cilento VOO and olive oil pomace.

Compound	Virgin olive oil		Olive oil pomace	
	"La Pepa"[a]	"Severini"[a]	"La Pepa"[a]	"Severini"[a]
(1) Gallic acid	43.8 ± 0.99	34.3 ± 1.0	11.4 ± 0.35	12.6 ± 0.65
(2) Hydroxytyrosol	41.3 ± 1.04	37.0 ± 1.31	10.4 ± 0.24	8.4 ± 0.56
(3) Tyrosol	23.8 ± 0.62	34.6 ± 1.52	20.7 ± 0.56	21.6 ± 0.98
(4) Caffeic acid	20.7 ± 0.89	30.0 ± 1.50	13.5 ± 1.04	6.7 ± 0.66
(5) Syringic acid	15.1 ± 0.74	19.2 ± 0.87	–	–
(6) Oleuropein	140 ± 2.99	120.4 ± 2.01	83.0 ± 3.60	81.7 ± 2.40
(7) Ligstroside aglycone	23.8 ± 0.7	35.0 ± 1.21	31.1 ± 1.53	27.1 ± 1.55
(8) Oleuropein aglycone	24.9 ± 0.9	19.9 ± 0.95	24.0 ± 1.21	23.3 ± 1.63
(9) Ferulic acid	4.6 ± 0.8	6.2 ± 0.45		12.6 ± 0.61
Vanillic acid	–	–	10.4 ± 0.66	8.8 ± 0.65

[a] Mean ± SD (standard deviation of recovery studies) of three determinations by the HPLC–DAD method.

Table 2
Total phenol content and free-radical scavenging activity of the VOO, olive oil pomace, and leaves, from *O. europaea* from Cilento.

Extract	Phenol content[a] (mg/kg)[b]	DPPH test EC_{50}[c]
VOO "La Pepa"	350 ± 4.2	42.3 (41.1–43.4)[d]
VOO "Severini"	343 ± 5.0	40.9 (40.1–42.3)[d]
Olive oil pomace "La Pepa"	207.4 ± 10.5	99.7 (99.6–99.8)[d]
Olive oil pomace "Severini"	210 ± 8.2	101.3 (101.2–101.5)[d]
Leaves	381.4 ± 7.4	37.6 (36.9–38.3)[d]
α-tocopherol[e]		10.1 (8.8–11.4)[d]
Oleuropein		45.1 (43.6–46.6)[d]

[a] Mean ± SD of three determinations by the Folin–Ciocalteu method.
[b] Oleuropein equivalents.
[c] In units of μg of extract or compound/ml.
[d] 95% confidence limits.
[e] Positive control.

while tyrosol, caffeic acid, and gallic acid in olive oil pomace, respectively. The concentration of these substances is largely affected by agronomic and technological conditions of VOO production. Cultivar, ripening stage, geographic origin of olive and olive trees irrigation can modify VOO phenolic composition. For this reasons the range in the averaged concentration of these VOO compounds is very high (Servili & Montedoro, 2002). The total phenolic derivatives content is in agreement with other Mediterranean oil (Esti, Contini, Moneta, & Sinesio, 2009; Franconi et al., 2006), also if Cilento VOO is characterised by a simple phenols major amount.

3.3. Isolation of compounds

The air-dried powdered leaves of *O. europaea* were defatted with petroleum ether and successively extracted for 48 h with $CHCl_3$, $CHCl_3$–MeOH (9:1), and MeOH. Each extract was tested for antioxidant activity and for the quantitative determination of total phenols. The methanol extract was the most active and exhibited an IC_{50} value of 37.6 μg/ml corresponding to 381.4 ± 1.9 μg/ml of total phenol content, while all the other extracts did not show any activity. On the basis of this result, the methanol extract was successively chromatographed on Sephadex LH-20 column, followed by RP-HPLC to give pure phenolic compounds as reported in Section 2.5. Analogously, olive oil pomace was defatted and extracted with solvent at increasing polarity. The methanol extract was fractionated over Sephadex LH-20 obtaining six groups that were tested in the DPPH assay. Only group 2 demonstrated antioxidant activity (EC_{50} 67.6 μg/ml) and was subjected to HPLC analysis as reported in Section 2.6.

3.4. Antioxidant activity

The model of scavenging stable radical DPPH (Picerno et al., 2003) is a widely used method to evaluate antioxidant capacities of natural products, and it has been used for olive oil as well as individual antioxidant polyphenols (Espin, Soler-Rivas, & Wichers, 2000). In the present work, we evaluated the antioxidant activity of polar extracts of VOO, olive oil pomace, and leaves, from *O. europaea* from National Park of Cilento. As shown in Table 2, the leaves and the VOO extracts elicited a significant free-radical scavenging effect at 10 min; the effect were concentration-dependent, so the EC_{50} value of the extracts were calculated as 37.6 μg/ml (36.9–38.3 μg/ml, 95% confidence limits), 42.3 μg/ml (41.1–43.4 μg/ml), and 40.9 μg/ml (40.1–42.3 μg/ml), for leaves, VOO "La Pepa", and VOO "Severini", respectively, with respect to α-tocopherol (EC_{50} = 10.1 μg/ml; 8.8–11.4 μg/ml) used as positive control. This strong free-radical scavenging activity was correlated to their high level of total phenols content determined by the Folin–Ciocalteu method (381.4 ± 7.4 μg/mg, 350 ± 4.2 μg/mg, and 343 ± 5.0 μg/mg, respectively) and expressed as oleuropein equivalent. On the contrary, the olive oil pomace extract showed a lower antioxidant activity in the DPPH test correlated to a minor total phenolic content (Table 2).

Since the secoiridoid constituents represent more than 50% of VOO from National Park of Cilento phenolic fraction, we also investigated the possible protective effect of the secoiridoids against reactive oxygen species (ROS) both in vitro and in Caco-2 cells.

Superoxide anion is one of the most aggressive ROS products in human organisms. Phenolic compounds like flavonoids have been shown to scavenge free radicals and their vasoprotective action has been associated with this particular property. Using an enzymatic biological generator of superoxide anion we have studied the free-radical scavenging activity of VOO secoiridoids. All investigated compounds inhibited the development of colour produced during the reaction of superoxide anion with NBT, in a moderate range of activity (Table 3).

In an effort to exclude the hypothesis that the superoxide anion scavenging activity was a result of an inhibition of XOD enzymatic systems, we have investigated the activity of the secoiridoids as inhibitors against the product of uric acid from xanthine in the oxidation reaction catalyzed from XOD. Only oleuropein showed a moderate activity (IC_{50} 63.98 μM) that partially explained the reduced production of the superoxide anion, while the other compounds had no activity (Table 3).

To investigate ROS-induced cytotoxic effects on differentiated Caco-2 cells, we added increasing amounts of H_2O_2 to the medium, bathing the apical side of the cells and after incubation we evaluated the cellular alterations. The overall cellular injury was measured by means of the neutral red assay. Incubation of cells in the presence of millimolar concentrations of H_2O_2 resulted in a significant decrease in Caco-2 viability; after 20 h of treatment with

Table 3
Superoxide anion scavenging activity and xanthine oxidase activity inhibition of secoiridoids[a].

Compounds	Superoxide anion scavenging activity IC_{50} (μM)	Xanthine oxidase activity inhibition IC_{50} (μM)
Ligstroside aglycone	94.93 ± 0.42	>100
Oleuropein	56.35 ± 1.18	63.98 ± 2.13
Oleuropein aglycone	82.33 ± 1.06	>100
Dihydroresveratrol	60.88 ± 1.12	>100

[a] Values are means of three repetition ± SD.

Table 4
Effect of oleuropein, oleuropein aglycone, ligstroside aglycone on H_2O_2-induced cytotoxicity in Caco-2 cells[a].

Compounds	Concentration	Cell viability (%)
Control	–	100
H_2O_2	+10 mmol/l	75
Ligstroside aglycone	+500 μmol/l	87
	+250 μmol/l	81
Oleuropein	+500 μmol/l	88
	+250 μmol/l	–
Oleuropein aglycone	+250 μmol/l	97
	+125 μmol/l	89

[a] All the variables were tested in three independent cultures for each experiment and each experiment was repeated three times ($n = 9$). Values are means ± SD. Level of significance: $P < 0.05$.

10 mmol/l H_2O_2 we observed about 25% loss of cell viability. Then, this marker was used to verify the protective effect of VOO secoiridoids against H_2O_2-induced injury to the intestinal Caco-2 cells. When cells were pretreated with oleuropein aglycone before being challenged with 10 mmol/l H_2O_2, no decrease in cell viability was observed, indicating that oleuropein aglycone at a dose of 250 μmol/l suppresses the H_2O_2-induced toxicity. At the same dose, ligstroside aglycone was inactive (Table 4).

We also studied the cytotoxic effect when the oxidative stress was induced by XOD and its substrate xanthine. The cells were pre-incubated in the presence of 200 mmol/l xanthine and increasing concentrations of XOD and a marked decrease of neutral red was observed compared with the control. The pretreatment of cells at little as 125 μmol/l with oleuropein aglycone completely prevented XOD-induced loss of viability whereas ligstroside aglycone showed activity at 250 μmol/l (Table 5).

Thus, findings obtained in this study demonstrated that "Cilento" VOO possess antioxidant/free-radical scavenging properties, which are very likely due to the presence of high contents of phenolic compounds. The polyphenols of olive oil proved to be effective in different tests as free-radical scavengers showing weak to moderate activities dependent on their structural features (Carrasco-Pancorbo et al., 2005; Gordon, Paiva-Martins, & Almeida, 2001). As expected, compounds with the presence of a 3,4-dihydroxy moiety linked to an aromatic ring were more active than those with only one hydroxyl group (Morello, Voureola, Romero, Motilva, & Heinonen, 2005); the glycosidation decreased the antioxidant activity. Our studies confirmed these findings: in fact, oleuropein aglycone, a hydroxytyrosol derivative, was more active than ligstroside aglycone and oleuropein glycoside. Moreover these findings suggest that VOO could exert a protective effect against those pathologies whose etiology has been related to ROM-mediated injuries.

Table 5
Effect of oleuropein, oleuropein aglycone, and ligstroside aglycone on xanthine oxidase-induced cytotoxicity in Caco-2 cells[a].

Compounds	Concentration	Cell viability (%)
Control	–	100
XO	+10 U/l	80
Ligstroside aglycone	+500 μmol/l	86
	+250 μmol/l	74
Oleuropein	+500 μmol/l	77
	+250 μmol/l	–
Oleuropein aglycone	+250 μmol/l	96
	+125 μmol/l	93
	+100 μmol/l	89

[a] All the variables were tested in three independent cultures for each experiment and each experiment was repeated three times ($n = 9$). Values are means ± SD. Level of significance: $P < 0.05$.

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