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Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves

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Abstract

The purpose of this study was to identify the main phenolic compounds present in an olive leaf extract (OL) in order to delineate the differential antioxidant activities of these compounds through the extent of their abilities to scavenge the $ABTS^{\bullet+}$ radical cation and to clarify the structural elements conferring antioxidant capacity in aqueous systems. The results show that the relative abilities of the flavonoids from olive leaf to scavenge the $ABTS^{\bullet+}$ radical cation are influenced by the presence of functional groups in their structure, mainly the B-ring catechol, the 3-hydroxyl group and the 2,3-double bond conjugated with the 4-oxo function. For the other phenolic compounds present in OL, their relative abilities to scavenge the $ABTS^{\bullet+}$ radical cation are mainly influenced by the number and position of free hydroxyl groups in their structure. Also, both groups of compounds show synergic behaviour when mixed, as occurs in the OL.

Keywords: Olive leaf extract (OL); Oleuropein; Flavonoids; Phenolics; ABTS radical cation; Antioxidant; Radical scavenger

1. Introduction

There is a rising interest in natural antioxidants as bioactive components of foods. The protective effects of diets rich in fruit and vegetables against cardiovascular diseases and certain cancers have been attributed partly to the antioxidants contained therein, particularly to phenolic compounds (Hertog, Feskeens, Hollman, Katan & Kromhout, 1993). Flavonoids are a widely distributed group of polyphenolic compounds, identified in recent years as antioxidants in various biological systems (Benavente-García, Castillo, Marin, Ortuño & Del Rio, 1997; Morel et al., 1993; Salah, Miller, Paganga, Tijburg, Bolwell & Rice-Evans, 1995; Saskia et al., 1996; Whang & Zheng, 1992).

The Mediterranean diet, rich in fresh fruits and vegetables, has been associated with a low incidence of cardiovascular disease and cancer, partly because of its high proportion of bioactive compounds such as vitamins, flavonoids and polyphenols. The major lipid component of such a diet is the drupe-derived olive oil that can be distinguished from other seed oils by the peculiar composition of its non-triglyceride fraction. In fact, several minor components, including polyphenols, grant the oil its particular taste and aroma.

Historically, olive leaf has been used as a folk remedy for combating fevers and other diseases, such as malaria. Several reports have shown that olive leaf extract had the capacity to lower blood pressure in animals (Samuelsson, 1951) and increased blood flow in the coronary arteries (Zarzuelo, 1991), relieved arrhythmia and prevented intestinal muscle spasms. The bitter compound oleuropein, the major constituent of the secoiridoid family in the olive (*Olea europaea* L.) trees, has been shown to be a potent antioxidant endowed with antiinflammatory properties. Oleuropein was discovered in 1908 by Bourquelot and Vintilesco, and its structure (see Table 2 below) was specified as being that of a heterosidic ester of elenolic acid and dihydroxyphenylethanol (Pannizzi, Scarpati & Oriente, 1960). Oleuropein has antimicrobial activity against viruses, retroviruses, bacteria, yeasts, fungi, molds and other parasites (Aziz, Farag, Mousa & Abo-Zaid, 1998; Juven & Henys, 1972; Koutsoumanis, Tassou, Taoukis &

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Nychas, 1998; Tassou & Nychas, 1995). Other clinical effects of oleuropein are the potentiation of cellular and organismal protection through the macrophage-mediated response (Visioli, Bellomo & Galli, 1998; Visioli, Bellosta & Galli, 1998) and the inhibition of platelet agregation and eicosanoid production (Petroni, Blasevich, Salami, Papini, Montedoro & Galli, 1995).

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of oxygen free radicals. The interaction of these species with molecules of a lipidic nature produces new radicals: hydroperoxides and different peroxides (Aust & Sringen, 1982; Pryor, Lightsey & Prier, 1982; Torel, Cillard & Cillard, 1986). This group of radicals (superoxide, hydroxyl and lipoid peroxides) may interact with biological systems in a clearly cytotoxic manner. In this respect, flavonoids, phenols and oleuropeosides have been shown to posses an important antioxidant activity towards these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors, Hellers, Michel & Saran, 1990a, 1990b; Visioli, Bellomo et al., 1998; Visioli, Bellosta et al., 1998). For flavonoids, three structural groups are important for determining their radical scavenging and/or antioxidative capacity: the *o*-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals; the 2,3-double bond conjugated with a 4-oxo function, responsible for electron delocation from the B-ring and the presence of both 3- and 5-hydroxyl groups for maximal radical-scavenging capacity and strongest radical absorption (Bors et al., 1990a; 1990b). For oleuropeosides and the other phenols present in OL, it is mainly the *o*-dihydroxy (catechol) structure present in their moieties which confers antioxidant properties to these compounds.

Obviously, the antioxidant capacity of any phenolic compound will be determined by a combination of these structural elements. However, this capacity will not be similar or show the same degree of effectiveness towards each of the above mentioned radicals but will depend on the different action mechanisms which take place in each particular case. These mechanisms are influenced by structural factors other than those described, such as the presence or absence of glycosidic moieties in the polyphenol, the glycosylation site, number and position of the free and sterified hydroxyls, etc.

The purpose of this study was to identify the main phenolic compounds present in an OL obtained from *Olea europaea* L. leaves of five cultivars, Villalonga, Alfafarenca, Picual, Cornicabra and Blanqueta from the regions of Andalucia and Murcia (south of Spain), in order to delineate the differential antioxidant activities of these compounds by the extent of their abilities to scavenge the $ABTS^{\bullet+}$ radical cation and to establish the synergic behaviour of these compounds in the OL.

2. Materials and methods

2.1. Chemicals

Oleuropein, verbascoside, tyrosol, hydroxytyrosol, apigenin-7-glucoside, luteolin-7-glucoside, diosmetin-7-glucoside, luteolin, diosmetin, rutin and catechin were obtained from Extrasynthèse (Genay, France). Vanillin, vanillic acid, caffeic acid, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt), Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid) and manganese dioxide were obtained from Sigma Chemical Co. (Madrid, Spain). OL was manufactured and supplied by Furfural Español S.A. (Alcantarilla, Murcia, Spain).

2.2. Chromatographic analysis and identification of phenolic compounds

For the quantification of phenolics in the leaf extract of *Olea europaea* L. (OL), the extract was dissolved in dimethylsulfoxide (DMSO) in the ratio of 5 mg/ml; this solution was filtered through a 0.45 μm nylon membrane. The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C_{18} LiChrospher 100 analytical column (250×4 mm i.d.) with a particle size of 5 μm (Merck, Darmstadt, Germany) thermostated at 30°C. The flow rate was 1 ml/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) during 20 min; it changed to 50% (A) and (B) in 20 min (40 min, total time); in 10 min it changed to 20% (A) and 80% (B) (50 min, total time), after reequilibration in 10 min (60 min, total time) to initial composition.

Phenolic compounds in OL were identified by comparation of their retention times with the corresponding standard and by their UV spectra obtained with the diode array detector.

2.3. Antioxidant capacity

The antioxidant capacity was measured used the method of Miller, Sampson, Candeias, Bramley and Rice-Evans (1996), based on the abilities of different substances to scavenge the $ABTS^{\bullet+}$ radical cation compared with a standard antioxidant (Trolox) in a dose–response curve.

$ABTS^{\bullet+}$ radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt) through manganese dioxide (MnO_2) on a Whatman no. 5 filter paper. Excess of MnO_2 was removed from the filtrate by passing it through a 0.45 μm nylon syringe

filter. This solution was then diluted in 5 mM phosphate buffered saline (PBS) pH 7.4 to an absorbance of 0.70 (± 0.02) at 734 nm and pre-incubated at 30°C prior to use. Fresh $ABTS^{\bullet+}$ radical cation solution was prepared each day. 2.5 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was prepared in PBS for use as stock standard. Fresh working standards were prepared daily by diluting 2.5 mM Trolox with PBS.

All oleuropeosides, flavonoids and phenols were dissolved in DMSO to a concentration of 50 μM. After addition of 1 ml of $ABTS^{\bullet+}$ solution to aliquots of Trolox or flavonoids (1–100 μl, depending on the activity of the particular compound) the solutions were vortexed for exactly 30 s and the absorbance at 734 nm was taken exactly 1 min after initiation of mixing in a Unicam spectrophotometer at 30°C. PBS blanks and DMSO blanks were run in each assay. The dose–response curve for Trolox consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation and was based on triplicate determinations. The activities of phenolics were assayed at four different concentrations which had been determined to be within the range of the dose–response curve. All the measurements were repeated on triplicate samples at these four concentrations. By reference to the Trolox dose–response curve, the mean Trolox equivalent antioxidant capacity (TEAC) value was derived for each phenolic compound and for whole OL.

3. Results and discussion

3.1. Identification of phenolic compounds present in OL

The HPLC profiles of phenolic compounds present in OL are shown in the Fig. 1. The retention times and abundance of the main compounds in OL are shown in Table 1. Five groups of compounds are present principally: oleuropeosides (oleuropein and verbascoside); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin and diosmetin); flavonols (rutin); flavan-3-ols (catechin) and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid).

The most abundant compound in OL is oleuropein, followed by hydroxytyrosol, the flavone-7-glucosides of luteolin and apigenin and verbascoside. Hydroxytyrosol is a precursor of oleuropein and verbascoside is a conjugated glucoside of hydroxytyrosol and caffeic acid. The chemical structures and the UV spectra of these five compounds are shown in the Table 2 and Fig. 2, respectively.

3.2. Antioxidant activity: $ABTS^{\bullet+}$ scavenging capacity

The abilities of different phenolic compounds from OL assayed to be scavenging the $ABTS^{\bullet+}$ radical cation in comparison with Trolox under defined conditions are shown in Table 3. This table shows that the most effective flavonoid for scavenging the $ABTS^{\bullet+}$ radical cation was the flavonol rhamnoglucoside rutin (TEAC 2.75 mM), followed by the flavan-3-ol catechin (TEAC 2.28 mM) and the flavone luteolin (TEAC 2.25 mM). These results confirm the importance of the flavonoid B-ring catechol structure (rutin, catechin and luteolin), the presence of a 3-hydroxyl free or glycosylated group (catechin and rutin), and the 2,3-double bond conjugated with a 4-oxo function (rutin and luteolin), which are responsible for electron delocation between the A and B flavonoid rings, stabilising the aroxyl radical after hydrogen donation when scavenging the $ABTS^{\bullet+}$ radical cation in the same form that occurs for $ABTS^{\bullet+}$ scavenging by carotenoids (Miller et al., 1996). The

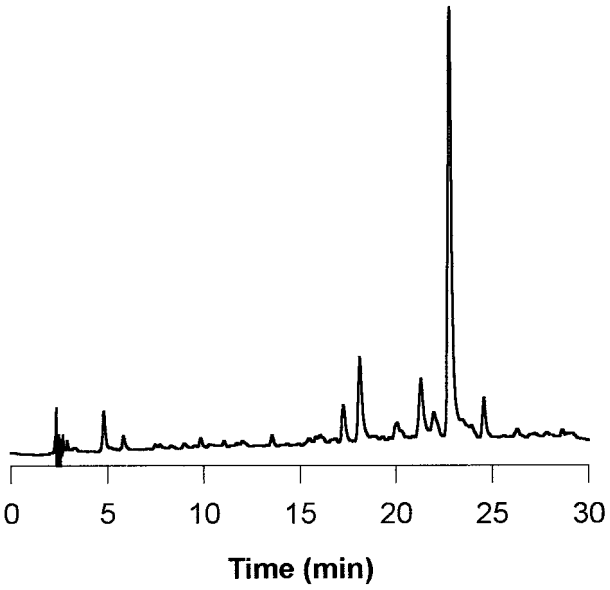
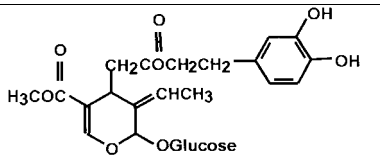
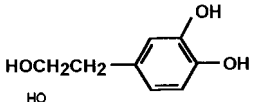
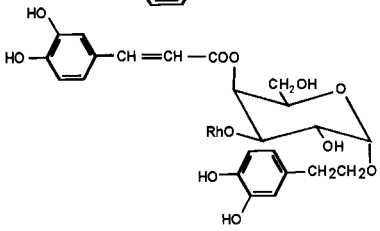
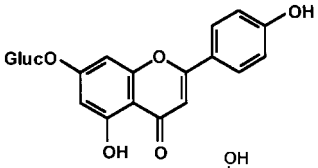
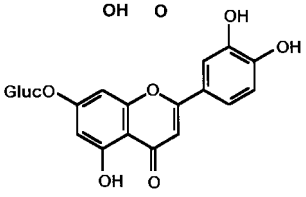


Fig. 1. HPLC chromatogram of olive leaf extract (OL), using a C_{18} Lichrospher 100 analytical column (250×4 mm i.d.), with an average particle size of 5 μm, thermostated at 30°C. The flow rate was 1 ml/min and the absorbance changes were monitored at 280 nm.

Table 1
Retention time and abundance of the main phenolic compounds present in olive leaf extract (OL) (absolute content dry basis)

Phenolics	Retention time (min)	% Absolute
Hydroxytyrosol	4.80	1.46
Tyrosol	5.83	0.71
Catechin	8.41	0.04
Caffeic acid	11.56	0.34
Vanillic acid	14.17	0.63
Vanillin	14.79	0.05
Rutin	17.22	0.05
Luteolin-7-glucoside	18.10	1.38
Verbascoside	20.06	1.11
Apigenin-7-glucoside	21.28	1.37
Diosmetin-7-glucoside	21.95	0.54
Oleuropein	22.76	24.54
Luteolin	28.61	0.21
Diosmetin	31.59	0.05

Table 2
Chemical structures of the most abundant phenolics in olive leaf extract (OL)

Phenolic compound	Chemical formula
Oleuropein	
Hydroxytyrosol	
Verbascoside	
Apigenin-7-glucoside	
Luteolin-7-glucoside	

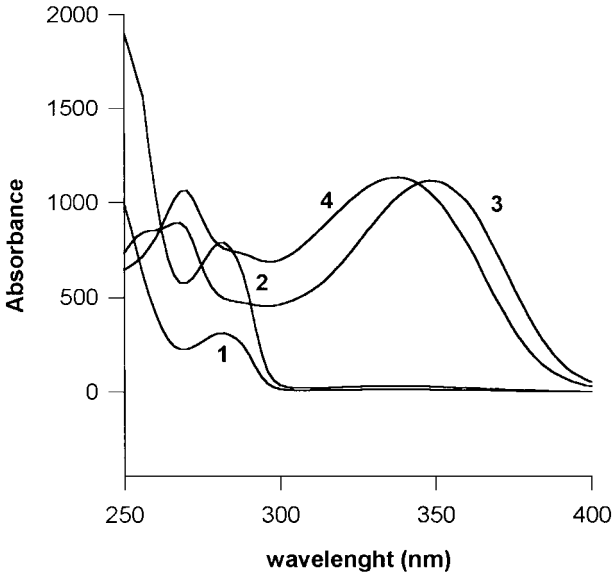


Fig. 2. Normalised UV-spectra (on the diode array detector, in the elution solvent of Fig. 1) of the main phenolic compounds present in the olive leaf extract: 1, oleuropein; 2, hydroxytyrosol; 3, luteolin-7-glucoside; 4, apigenin-7-glucoside.

ability shown by catechin, despite the absence of a 2,3-double bond conjugated with the 4-oxo function, confirms the importance of B-ring catechol and free 3-hydroxyl groups for maximal radical-scavenging capacity and strongest radical absorption (Benavente-García et al., 1997; Bors et al., 1990a, 1990b; Rice-Evans & Miller, 1996; Salah et al., 1995).

Removing the 3-hydroxyl group, as in luteolin, reduces the contribution to electron delocation and, consequently, the antioxidant activity; this reduction is less when such a hydroxyl is substituted that when it is absent (quercetin, TEAC 3.5 mM, vs rutin vs luteolin). In addition, the presence of a 2,3-double bond in the absence of a 3-OH group (luteolin and diosmetin) does not significantly increase the antioxidant capacity of flavonoids with respect to those which lack this double bond (eriodictyol and hesperetin with TEAC 2.11 and 1.36 mM, respectively) despite that the double bond increases conjugation in the structure. This phenomenon might be due to the strong intramolecular hydrogen bond between the 5-hydroxyl and the 4-one in the C ring which reduces the contribution of the carbonyl group to the electron delocation. Accordingly this effect has, apparently, a similar consequence for the antioxidant activity to that derived by removing the 4-one group as in catechin, in which the presence of a 3-hydroxyl group increases the electron delocation across the flavonoid structure.

With reference to the other important structural element described for the antioxidant activity of natural polyphenols, the number and location of their aromatic hydroxyl groups (Chen, Chan, Ho, Fung & Wang, 1996), it is important to note that the absence of B-ring catechol structure significantly reduces the antioxidant capacity, both in the case that the 4′-hydroxyl group is methylated (diosmetin and diosmetin-7-glucoside) and with the presence of a single hydroxyl group (4′-OH) in the B-ring (apigenin and apigenin-7-glucoside).

These structural considerations are similar for the other phenols present in OL; thus, the important difference in the antioxidant activity between hydroxytyrosol vs tyrosol and caffeic acid vs vanillic acid. In contrast to the flavonoids, the presence of a double bond conjugated with the catechol structure in caffeic acid gave no increase of the antioxidant activity with respect to the catechol structure in hydroxytyrosol, because the higher electronic density in the side chain reduced the possibility of electron delocation.

The glucosylation of the 7-hydroxyl group of flavones (luteolin-7-glucoside and diosmetin-7-glucoside) reduced the antioxidant activity respect to their aglycones (luteolin and diosmetin). The 7-O-glucosylation produces conformational changes in the flavonoid molecule that might make electronic delocation difficult as well as decreasing the electron donor capacity of the 7-hydroxyl group.

Table 3
Antioxidant activity of phenolics from olive leaf extract (OL)

Phenolic compound	Chemical formula	TEAC (mM)
Olive leaf extract		1.58 ± 0.06
Oleuropein	Table 2	0.88 ± 0.09
Verbascoside	Table 2	1.02 ± 0.07
Tyrosol	4-hydroxyphenylethanol	0.35 ± 0.05
Hydroxytyrosol	Table 2	1.57 ± 0.12
Apigenin-7-glucoside	Table 2	0.42 ± 0.03
Luteolin-7-glucoside	Table 2	0.71 ± 0.04
Diosmetin-7-glucoside	5,3′-dihydroxy-4′-methoxyflavone-7-*O*-glucoside	0.64 ± 0.09
Luteolin	5,7,3′,4′-tetrahydroxyflavone	2.25 ± 0.11
Diosmetin	5,7,3′-hydroxy-4′-methoxyflavone	1.42 ± 0.07
Rutin	5,7,3′,4′-tetrahydroxyflavone-3-*O*-rhamnoglucoside	2.75 ± 0.05
Catechin	5,7,3′,4′-tetrahydroxyflavan-3-ol	2.28 ± 0.04
Vanillin	4-hydroxy-3-methoxybenzaldehyde	0.13 ± 0.01
Vanillic acid	4-hydroxy-3-methoxybenzoic acid	0.67 ± 0.09
Caffeic acid	3,4-dihydroxybenzoic acid	1.37 ± 0.08

Antioxidant activity of oleuropein is mainly due to the hydroxytyrosol moiety in its structure. This ability to scavenging the $ABTS^{\bullet+}$ radical cation in comparison with hydroxytyrosol is lower due to the increased molecular weight; however, the oleuropein structure makes this reduction of scavenging ability (TEAC mM ratio, 1.7) lower than the theoretical linear value according to the molecular weight ratio (MW ratio, 3.3). This behaviour is similar between verbascoside and precursors, hydroxytyrosol and caffeic acid. In this case, the presence of two catechol structures increases the theoretical TEAC mM value of verbascoside corresponding to the MW ratios.

The other factor that determines the antioxidant potential of phenolic compounds is the stability of the aroxyl radical formed, not taken into account in the method here described. However, the aroxyl radical species of the OL mono and polyphenols have molecular structures capable of an extensive electron delocation, which is a prerequisite for radical stabilization, generating multiple mesomeric structures. The decay rate constants of flavonoid aroxyl radicals generated by interrelation with other radicals show that all the most stable aroxyl radicals, without exception, contain the 3′,4′-catechol B-ring substitution pattern. All other polyphenolic compounds form far less stable aroxyl radicals (Benavente-García et al., 1997; Bors et al., 1990b; Bors & Saran, 1987).

The whole OL showed a TEAC value of 1.58 mM, increasing 72% with respect to the theoretical value obtained from the pooled average of individual TEAC (Table 3). This result suggests that olive phenolics show a synergic behaviour in their radical scavenging capacity when mixed, as occurs in the OL.

The sequence of relative radical scavenging abilities is: rutin > catechin ≅ luteolin > OL ≅ hydroxytyrosol > diosmetin > caffeic acid > verbascoside > oleuropein > luteolin-7-glucoside ≅ vanillic acid ≅ diosmetin-7-glucoside > apigenin-7-glucoside > tyrosol > vanillin. The most active flavonoids, rutin, catechin and luteolin had antioxidant activities almost to 2.5 times those of vitamin C and E (TEAC = 1.12 and 1.10 mM, respectively) and in the order of lycopene (TEAC = 2.9 mM) (23). It is significative that OL had a antioxidant activity higher than vitamin C and E, due to the synergy between flavonoids, oleuropeosides and substituted phenols.

In conclusion, this paper demonstrates that flavonols, flavans-3-ols and flavones with catechol structures are the most efficient olive phenolic compound quenchers for the $ABTS^{\bullet+}$ radical cation and this ability is greater as more free hydroxyl groups are present in the flavonoid structure. Also, the flavonoids, oleuropeosides and substituted phenols show a synergic behaviour in mixed form, as occurs in a characteristic olive leaf extract with a high content of oleuropein and these active polyphenols.

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