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Contribution of Flavonoids to the Overall Radical Scavenging Activity of Olive (*Olea europaea* L.) Leaf Polar Extracts

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The contribution of flavonoids to the overall radical scavenging activity of olive leaf polar extracts, known to be good sources of oleuropein related compounds, was examined. Off line and on line HPLC-DPPH• assays were employed, whereas flavonoid content was estimated colorimetrically. Individual flavonoid composition was first assessed by RP-HPLC coupled with diode array and fluorescence detectors and verified by LC−MS detection system. Olive leaf was found a robust source of flavonoids regardless sampling parameters (olive cultivar, leaf age or sampling date). Total flavonoids accounted for the 13−27% of the total radical scavenging activity assessed using the on line protocol. Luteolin 7-*O*-glucoside was one of the dominant scavengers (8−25%). Taking into consideration frequency of appearance the contribution of luteolin (3−13%) was considered important, too. Our findings support that olive leaf, except for oleuropein and related compounds, is also a stable source of bioactive flavonoids.

KEYWORDS: Olive (*Olea europaea* L.) leaves; flavonoids; luteolin; radical scavenging activity; on line HPLC-DPPH•

INTRODUCTION

Olive (*Olea europaea* L.) leaf has gained the rising interest of the scientific and industrial community due to the numerous beneficial health properties, which were mainly attributed to oleuropein and related derivatives. Recently, Papoti and Tsimidou (*1*) stressed that, except for oleuropein, other secoiridoids and flavonoids should contribute to the overall antioxidant activity of olive leaf polar extracts. Indeed, although flavonoids, such as free or bound forms of luteolin, are among the major constituents of these extracts (*1*−*9*), their contribution to the overall antioxidant potency of this plant material has not been established yet.

The present study was undertaken in order to investigate the extent to which the presence of flavonoids affects the overall radical scavenging potential of olive leaf extracts. Estimation of flavonoid content was based on a metal−flavonoid complexing reaction, whereas the activity of the examined extracts was followed by the DPPH• assay. Separation and identification of individual flavonoids was accomplished using HPLC−DAD and HPLC−DAD−MS. An in house validated on line HPLC-DPPH• method allowed assessment of the participation of flavonoids to the overall antioxidant activity of the extracts. Activity-guided fractionation of plant extracts is a time-consuming, labor demanding, expensive process that cannot ensure avoidance of loss in responsible compounds. In addition, lack or expense of suitable standards have made high throughput activity evaluation assays such as hyphenated HPLC radical scavenging procedures valuable tools in the parallel examination of compositional and activity studies of natural extracts (*10*) as those under examination in the present one.

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MATERIALS AND METHODS

Chemicals. Morin and DPPH• radical (1,1-diphenyl-2-picrylhydrazyl, 90%) were from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride ($AlCl_3$) was purchased from Panreac Quimica (Barcelona, Spain). Acetic acid was provided by Merck (Darmstadt, Germany). HPLC grade methanol (MeOH) and acetonitrile (ACN) were from Scharlau (Barcelona, Spain). All other common reagents and solvents were of the appropriate purity from various suppliers.

Leaf Samples. The plant material was chosen from the olive leaf collection reported in ref *1*, wherein detailed information on sampling parameters and postharvest treatment are described. Briefly, leaves (mature from one year old shoots) of selected cultivars (cv.) [Greek: Adramatiani, Amfissis, Chondrolia Chalkidikis (Chondrolia Ch.), Kalamon, Kolovi, Koroneiki, Kothreiki, Megaritiki, Tsounati, Vassilikada. Spanish: Picual. Italian: Frantoio] were sampled from trees of an experimental olive orchard (Agios Mamas, Chalkidiki, Greece) in December 2006. Repetition of sampling for Adramatiani, Amfissis, Chondrolia Ch., Koroneiki and Vassilikada cv. was conducted in February 2008. In addition, leaves of different age (new-season and mature ones from one year old shoots, old season from two year old shoots and yellow ones) were sampled from one randomly selected tree (Chondrolia Ch. cv.; experimental orchard Aristotle University of Thessaloniki, Greece) in October 2007 and April 2008.

Leaf Extract Preparation. Extracts were prepared in triplicate by extracting 0.25 g of lyophilized leaf material with 10 mL of MeOH in an

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ultrasonic bath for 5 min at room temperature. The extracts were then filtered using ordinary filter paper and combined before being brought up to dryness. Dry extracts were redissolved in MeOH for HPLC, flavonoid content determination and antioxidant activity studies. HPLC analytical samples were filtered through 0.45 μm PTFE filter (Waters, Milford, MA) just before injection.

Determination of Flavonoid Content (FL). Flavonoid content was estimated according to a validated protocol (*11*), which is based on flavonoids−Al(III) complexation. An aliquot (0.1 mL) of an aluminum chloride solution (2% aluminum chloride in 5% acetic acid in methanol) was added to 1 mL of the test solution and subsequently 1.4 mL of 5% acetic acid in methanol. The tested aliquot contained ~1000 μg of dry extract. The mixture was left for 30 min at room temperature, and thereafter the absorbance was measured at 415 nm against a control. Absorbance measurements were corrected by subtracting initial sample absorbance at 415 nm. Flavonoid content results (mean value of two measurements) were expressed as micrograms of FL per gram of dry leaf through a morin calibration curve. The repeatabilities of measurement calculated for a morin standard solution and an extract were found to be satisfactory (CV % = 1 for both, $n = 5$).

HPLC Analysis of Phenolic Compounds. The HPLC system and software facilities used are explicitly described in ref *1*. Phenolic monitoring was accomplished at 245, 280, and 335 nm using a diode array (DAD) system and at 280 nm excitation/320 nm emission using a fluorescence detector. The chromatographic separation was carried out on a Chromolith RP-18e (100 × 4.6 mm) column (Merck, Darmstadt, Germany). Elution was performed using 3% acetic acid in water (solvent A) and ACN (solvent B) with the following linear gradient: 0−1 min, 4% B; 1−26 min, 4−30% B; 26−36 min, 30−60% B; 36−46 min, 60−98% B; 46−50 min, 98% B; 50−60 min, 98−4% B. The flow rate and the injection volume were 0.9 mL/min and 10 μL, respectively.

LC−MS Analysis. The HPLC 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 chromatographic separation was carried out on a Discovery C18 (250 mm × 4.6 mm i.d., 5μm) column (Supelco, Bellefonte, PA). Elution was performed using 0.1% acetic acid in water (solvent A) and ACN (solvent B) with the following linear gradient: 0−20 min, 5−25% B; 20−40 min, 25−50% B; 40−50 min, 50−80% B; 50−60 min, 80−5% B. The flow rate and the injection volume were 0.6 mL/min and 10 μL, respectively. ESI-MS in the negative mode was performed using an Agilent G2455A ion trap mass spectrometer equipped with Agilent software. The collected samples were dissolved in acetonitrile and infused in the ESI source with flow rate 100 μL/min. Operating conditions were as follows: accumulation time, 300 ms; dry temperature, 350 °C; capillary voltage, 3500 V; nebulizer, 30 psi; dry gas, helium at 8 L/min. Ion trap full scan analysis was conducted from m/z 50 to 1300 with an upper fill time of 200 ms. The scan mass spectra of the phenolic compounds were measured from m/z 50 up to m/z 800.

Antioxidant Activity Studies. *DPPH$^\bullet$ Assay*. Radical scavenging activity of methanol extracts was determined via the DPPH$^\bullet$ assay based on a protocol elsewhere described (*12*). Briefly, an aliquot (2.9 mL) of a methanolic DPPH solution (0.1 mM) was transferred in a glass cuvette and then mixed with 0.1 mL of a methanol extract. Absorption at 516 nm (A_{516}) was recorded at the start of the reaction and after 30 min. Results were expressed as % inhibition = $[A_{516(t=0)} - A_{516(t=30)} \times 100/A_{516(t=0)}]$. DPPH$^\bullet$ inhibition was estimated for all leaf extracts on the same FL basis (aliquot tested contained ~3 μg FL expressed as morin). All determinations were performed in triplicate at room temperature, and data are given as the mean ± standard deviation.

On Line HPLC-DPPH$^\bullet$ Assay. The on line HPLC-DPPH$^\bullet$ scavenging assay was performed using an Agilent G13311A solvent delivery pump and a Bruker DAD detector (Bruker BioSpin, Rheinstetten, Germany). The samples were injected using an Agilent G1311A autosampler with a 100 μL loop. A Knauer K 120 HPLC pump (Berlin, Germany) was connected to the system for postcolumn addition of the DPPH$^\bullet$ methanolic solution. The adequate reaction of the eluents and DPPH$^\bullet$ was accomplished in a Teflon reaction coil (15 m × 0.3 mm i.d.) according to ref *13*. The DPPH$^\bullet$ solution (5×10^{-5} mM) was pumped to the eluents at a flow rate of 0.2 mL/min. The chromatographic separation was carried out on a Discovery C18 (250 mm × 4.6 mm i.d., 5 μm) column (Supelco, Bellefonte, PA). The flow rate and the injection volume were 0.6 mL/min and 20 μL, respectively. Elution was performed using 0.1% acetic acid in water (solvent A) and ACN (solvent B) with the following linear gradient: 0−20 min, 5−25% B; 20−40 min, 25−50% B, 40−50 min, 50−80% B, 50−60 min, 80−5% B. The separated analytes reacted postcolumn with the DPPH$^\bullet$ solution. Bleaching of the latter was recorded at 517 nm, whereas phenol monitoring was accomplished at 254, 280, and 335 nm. The flow diagram of the hyphenated system is illustrated in Figure S1 (Supporting Information).

Statistical Analysis. Statistical comparisons of the mean values for each experiment were performed by one-way analysis of variance (ANOVA), followed by the multiple Duncan test ($p < 0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The systematic examination of the impact of sampling parameters on the radical scavenging potential of olive (*Olea europaea* L.) leaves indicated that oleuropein is not always the main leaf component and that other related secoiridoids, along with verbascoside and flavonoids, become the major leaf constituents in certain phenological stages (*1*). In the same study it was also found that despite variability in the levels of individual phenolics, leaf is a rather robust source of antioxidants, in terms of its total polar phenol (TPP) content and antiradical potency. Therein, it was also stated that in view of future exploitation of olive leaf extracts the contribution of individual compounds to the overall antioxidant activity is expected to add to the knowledge needed. Consequently, the present study focuses on the contribution of individual flavonoids—well-known radical scavengers but less studied components of olive leaf extracts—to the overall antioxidant activity of the plant material.

At first, it was considered important to evaluate the flavonoid content of olive leaf polar extracts. For its quantitative estimation, among the various recommended protocols, we employed an assay based on flavonoids−Al(III) complexation (*11*, *14*). Taking into account stoichiometry, measurement at 415 nm was accomplished after acid addition to the reaction mixture. The presence of acid precludes codetermination of complexes formed via ortho-dihydroxyl groups, so that 3-hydroxy or 5-hydroxy 4-keto groups are the only responsible moieties for complex formation. This was considered of major importance since hydroxytyrosol, oleuropein, cinnamic acids and corresponding derivatives (e.g., 3,4-DHPEA-EDA, verbascoside) bearing ortho-dihydroxyl groups were also present in the examined extracts.

Olive leaf was found to contain significant amounts of flavonoids, regardless of "cultivar" or "sampling date" (**Table 1**). Variability among cultivars was not larger than that found "within cultivar" (**Table 2**), so that leaf can be regarded not only as a stable source of TPP (*1*) but also as a stable source of flavonoids. However, on the same FL basis, the DPPH$^\bullet$ scavenging activity of the extracts was found to vary more than when expressed on the same TPP content (**Tables 1** and **2** and Tables 1 and 2 of ref *1*). It can be suggested that FL is a less safe criterion for the selection and appreciation of olive leaf batches for further usage in comparison to TPP content. Moreover as shown in **Table 3** all types of leaves (new, mature, old, yellow) for both sampling periods (October 2007 and April 2008 sampling) were good sources of flavonoids. Variability in antioxidant activity of these extracts was not distinctive.

RP-HPLC of the studied extracts revealed qualitatively similar phenolic profiles at 245, 280, 335 nm and 280/320 nm (excitation/emission) for all samples tested. Fluorescence detection did not assist identification of flavonoids to the extent it was found useful for the detection of hydroxytyrosol derivatives. The same applied

Table 1. Flavonoid Content (FL) and DPPH• Radical Scavenging Activity of Methanol Extracts Prepared from Leaves Varying in Cultivar (December 2006 Sampling)[a]

cultivar/block B	flavonoid content: flavonoid−Al(III) complexation assay[b]	antioxidant activity on the same FL basis: DPPH • assay[c]
Adramatiani	2127 a,b,c (1714 b)	71 ± 2 f (59 ± 2 d)
Amfissis	3017 d (2325 c)	56 ± 3 d (30 ± 2 b)
Ch. Chalkidikis	2001 a,b,c (1166 a)	70 ± 3 f (79 ± 3 e)
Kalamon	1967 a,b	33 ± 1 b
Kolovi	1820 a	65 ± 5 e
Koroneiki	2336 c (2076 b,c)	32 ± 1 b (49 ± 2 c)
Kothreiki	2245 b,c	50 ± 2 c
Megaritiki	2959 d	52 ± 1 c,d
Tsounati	2890 d	56 ± 2 d
Vassilikada	2026 a,b,c (1874 b)	73 ± 4 f (22 ± 1 a)
Frantoio	1879 a	21 ± 1 a
Picual	2078 a,b,c	64 ± 2 e

[a] Data in parentheses concern sampling in February 2008. [b] Results are expressed as μg of morin/g of dry leaf, mean value of two measurements. [c] Results are expressed as % DPPH• inhibition ± standard deviation (n = 3). Values within the same column bearing different letters are significantly different ($p < 0.05$).

Table 2. Flavonoid Content (FL) and DPPH• Radical Scavenging Activity of Methanol Extracts Prepared from Chondrolia Ch. and Koroneiki Cv. Leaves (December 2006 Sampling) from Trees Belonging to Different Blocks (A, B and C) of the Experimental Orchard to Examine Variability within Cultivar

cultivar/block	flavonoid content: flavonoid−Al(III) complexation assay[a]	antioxidant activity on the same FL basis: DPPH •assay[b]
Chondrolia Ch./A	1885 a	78 ± 3 c
Chondrolia Ch./B	2001 a	70 ± 3 b
Chondrolia Ch./C	3596 b	54 ± 1 a
Koroneiki/A	2615 a	39 ± 3 b
Koroneiki/B	2336 a	32 ± 1 a
Koroneiki/C	3878 b	38 ± 1 b

[a] Results are expressed as μg of morin/g of dry leaf, mean value of two measurements. [b] Results are expressed as % DPPH• inhibition ± standard deviation (n = 3). Values within the same column bearing different letters are significantly different ($p < 0.05$).

Table 3. Flavonoid Content (FL) and DPPH• Radical Scavenging Activity of Methanol Extracts Prepared from Leaves Varying in Leaf Age

leaf age	flavonoid content: flavonoid−Al(III) complexation assay[a]	antioxidant activity on the same FL basis: DPPH •assay[b]
	October 2007 Sampling	
new	3880 b	54 ± 2 c
mature	4286 b	40 ± 2 a,b
old	4822 c	37 ± 1 a
yellow	3163 a	43 ± 2 b
	April 2008 Sampling	
new	2261 a	65 ± 3 c
mature	2999 b,c	48 ± 1 a
old	3269 c	47 ± 1 a
yellow	2641 a,b	56 ± 3 b

[a] Results are expressed as μg of morin/g of dry leaf, mean value of two measurements. [b] Results are expressed as % DPPH• inhibition ± standard deviation (n = 3). Values within the same column bearing different letters are significantly different ($p < 0.05$).

for detection at 245 nm. Chromatographic profiles at 335 nm allowed appraisal of the extent of variability among individual flavonoids. This variation is exemplified for two of the cultivars in Figure S2 (Supporting Information). Assessment of the contribution of individual active compounds to the overall potential of the extracts with emphasis on flavonoids was, thus, considered useful and was accomplished via an in house validated on line HPLC-DPPH• assay. In addition, LC−MS analysis of selected extracts was employed under the same elution conditions to verify the identity of the most reactive compounds toward the DPPH•. Using this system, peaks were recorded at selected wavelengths, i.e. 254, 280, 335, and 517 nm, which are appropriate for secoiridoids, simple phenols, flavonoids and DPPH• reduction monitoring, respectively. The majority of the main leaf phenolics were shown to scavenge the DPPH• radical, as is representatively depicted in **Figure 1**.

As suggested in the studies of Bandoniere and Murkovic (*15*), and Gioti and collaborators (*16*), the relative contribution of each antioxidant can be calculated as percentage of the total antiradical activity. In their view, the areas of negative peaks at 517 nm are used to evaluate the percent contribution of individual active constituents (total negative peak area: 100) to overall extract activity.

The results are shown in **Table 4**. As shown in **Table 4**, flavonoids contribute satisfactorily to the overall radical scavenging potential of the examined extracts (13−27%), under the experimental conditions. In detail, luteolin 7-*O*-glucoside, one of the major leaf components, was shown to be one of the most prevailing radical scavengers (8−25% to the total recorded activity). The contribution of luteolin (3−13%) to the overall antiradical potency of the extracts was also considered essential taking into consideration its high frequency of appearance, as this flavonol was present in almost every sample. Rutin, luteolin 7-*O*-rutinoside and apigenin 7-*O*-rutinoside that were found in some of the studied samples presented also a slight to moderate contribution. Other components, such as luteolin 4′-*O*-glucoside and apigenin glucoside, although found in noteworthy amounts in certain extracts, were found to be inactive. The latter was expected due to established flavonoid structure−activity relationships (*17*). The glucosylation of the active hydroxyl group of B ring, as in the case of luteolin 4′-*O*-glucoside, deprives the active ortho-dihydroxyl domain arising to a less efficient radical scavenger in comparison to luteolin and its 7-*O*-glucoside.

The above findings were in line with literature data presenting flavonoids as active radical scavengers (*18*). In particular, several olive leaf flavonoids such as rutin, quercetin, luteolin and its glucosides are reported to be efficient radical scavengers, some presenting superiority in off line determinations in comparison to oleuropein and/or hydroxytyrosol (*19*−*21*). Furthermore, their antiradical potency has been also supported in relevant on line antioxidant assessment studies (*13*, *15*, *22*).

Among the rest of olive leaf phenolics, hydroxytyrosol, oleuropein and relevant derivatives, as well as verbascoside, were also shown to contribute to the overall antioxidant activity of the examined extracts. Specifically, hydroxytyrosol, present in all examined samples, showed an intense activity in agreement with numerous published data. This compound has been considered to be one of the most important and bioactive olive phenolics, possessing among others free radical scavenging capacity (*23*). The contribution of other hydroxytyrosol derivatives to the overall antioxidant potency was also observed. The on line DPPH• application allowed the discrimination of the contribution of hydroxytyrosol glycoside and hydroxytyrosol acetate, that would have been overlooked on account of their minor presence in the extracts under study. Hydroxytyrosol glucoside has been known to exhibit a relatively high free radical scavenging activity (~60% higher off line DPPH• inhibition than that of Trolox) (*24*), whereas the antiradical activity of hydroxytyrosol acetate has been reported to be efficient but lower than that of hydroxytyrosol (*19*, *25*). Total hydroxytyrosol and derivatives contribution (expressed as total negative peak area at 517 nm) varied

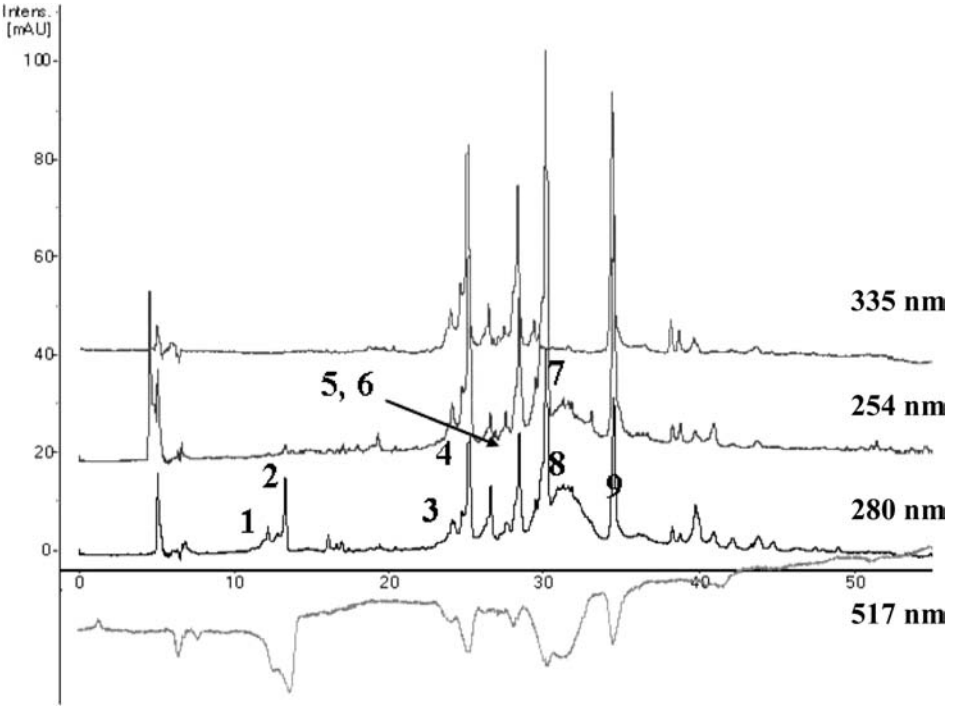

Figure 1. On line HPLC-DPPH• chromatograms at 254, 280, 335, and 517 nm: (1) hydroxytyrosol glucoside, (2) hydroxytyrosol, (3) verbascoside, (4) luteolin 7-*O*-glucoside, (5) luteolin 4′-*O*-glucoside, (6) unknown compound 1, (7) oleuropein, (8) oleuropein derivative, (9) luteolin.

Table 4. Percent Contribution of Individual Constituents to the Overall Radical Scavenging Activity of Olive Leaf Polar Extracts

	% contribution of individual constituents																
	Chondrolia Ch.																
	new		mature		old		yellow	Adramatiani[a]		Amfissis[a]	Chondrolia Ch.[a]		Koroneiki[a]		Vassilikada[a]		
constituents	2007	2008	2007	2008	2007	2008	2007	2006	2008	2006	2006	2008	2006	2008	2006	2008	range
hydroxytyrosol glucoside	8	8		10	6		7			4			6	7	7	15	4−15
hydroxytyrosol	18	22	25	26	20	31	5	26	24	10	24	25	20	32	14	20	5−32
hydroxytyrosol acetate											6						6
hydroxytyrosol and derivatives	26	30	25	36	26	31	12	26	24	14	30	25	26	39	21	35	12−39
oleuropein	18	9	21			8	15	9	14	35	11	21	12		22	38	8−38
oleuroside										7							7
oleuropein aglycon					26	15				9	3		4		4		3−26
oleuropein derivatives	29	22	19	30				35	25		26	19	27	33	23		19−35
secoiridoids	47	31	40	30	26	23	15	44	39	51	40	40	43	33	49	38	15−51
verbascoside	3	13	11	13	21	18	17	10	13	17	8	11				9	3−18
rutin					5								8	9			5−9
luteolin 7-*O*-rutinoside										5					4		4−5
luteolin 7-*O*-glucoside	10	16	14	12	11	17	25	11	13	8	13	14	12	10	13	9	8−25
apigenin 7-*O*-rutinoside							7				2				2		2−7
luteolin	13	6	5	6	8	7		5	3		6	5	7	4	5	6	3−13
flavonoids	23	22	19	18	24	24	32	16	16	13	21	19	27	23	24	15	13−27
unknown 1	3	4	5	3	4	4		4	6	6		5	5	4	4	3	3−6
unknown 2							22										
unknown 3							3										
unknown 4									3								

[a] Mature.

among extracts accounting for ∼12−39% of the total radical scavenging activity.

Oleuropein is an active olive phenolic having a well documented antiradical potential (*26*), which has been off line estimated to be from almost equal to slightly lower in comparison to that of hydroxytyrosol, depending on the experimental conditions (*19*, *25*). Apart from oleuropein, data shown in **Table 4**, the efficient contribution of other oleuropein related compounds to the overall activity of the extracts was not negligible. As depicted in **Figure 1**, peak 8, corresponding to an oleuropein derivative (m/z = 539), was responsible for 19−35% of the total activity. Oleuropein aglycon, found in few of the extracts, presented a moderate activity. This was in agreement with published data that assess oleuropein aglycon as a fairly active off line DPPH• scavenger (*27*). Oleuroside (oleuropein isomer), identified in one extract (Amfissis 2006), was shown to contribute to the overall activity irrespectively to its low level. As shown in **Table 4** the involvement of secoiridoids was considered significant (15−51% of total negative peak area at 517 nm).

Table 5. Negative Integrals at 517 nm Corresponding to DPPH• Bleaching Induced by an Equimolar Mixture of Selected Standards

compound	integral (517 nm)
hydroxytyrosol	1995
oleuropein	1437
rutin	1922
luteolin	1783
luteolin 7-*O*-glucoside	1485
luteolin 4′-*O*-glucoside	0

Moreover, verbascoside, present in relatively significant amounts in several samples, was found to contribute noticeably to the overall antioxidant capacity of the examined extracts. The latter is in line with literature findings presenting verbascoside as a 2−3-fold more active radical scavenger than hydroxytyrosol (*28*). In most of the examined samples traces of other than the previously described secoiridoids and flavonoids were also found, however their contribution was from zero to negligible and thus these compounds are not further discussed. An exception was observed in one extract (yellow 2007) in which unknown 2 (m/z = 325.5) was found to contribute to the overall antioxidant capacity to a significant extent (22%).

The above experimentation yields meaningful information if (a) response is dose dependent for each compound and (b) reactivity is justified by established structure activity criteria. Dose−response test for luteolin was quite satisfactory (y = 1696.3x + 3196.2, R^2 = 0.98), in line with similar studies (e.g., ref *22*) for this flavonoid, as well as for luteolin 7-*O*-glucoside. Moreover, equimolar mixtures of selected available standards, namely, hydroxytyrosol, oleuropein, rutin, luteolin and its 7-*O*- and 4′-*O*-glucoside, were prepared and analyzed in the HPLC-DPPH• setup under the same conditions. As it can be seen from the negative area values presented in **Table 5** for these standards, hydroxytyrosol was better than oleuropein in line with existing data. The order of activity among the tested flavonoids was also in line with established criteria (*17*, *19*, *20*, *29*, *30*). Thus, the negative peak size was considered as a good projection of the contribution of each and every compound of the extract to the overall activity.

Our findings support that olive leaf is not only a good source of secoiridoids but also a stable source of flavonoids, irrespectively of sampling parameters. Individual flavonoids along with hydroxytyrosol, oleuropein and related components, as well as verbascoside, were shown to contribute significantly to the overall leaf antioxidant potential. Luteolin 7-*O*-glucoside was identified as the main leaf constituent of the examined extracts and found to act as the dominant radical scavenger. Thus, although significant quantitative variability in individual compounds may be observed from sample to sample, olive leaf, as an entity, is expected to be a robust source of antioxidants throughout the year.

ABBREVIATIONS USED

ACN, acetonitrile; Chondrolia Ch., Chondrolia Chalkidikis; cv., cultivar; DAD, diode array; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MeOH, methanol; FL, flavonoid content; TPP, total polar phenols.

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Supporting Information Available: Figure S1, graphical illustration of the on line HPLC- DPPH• instrumentation, and Figure S2, RP HPLC chromatograms at 335 nm of leaf extracts. This material is available free of charge via the Internet at http://pubs.acs.org.

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