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ANALYSIS AND QUANTIFICATION OF FLAVONOIDIC COMPOUNDS FROM PORTUGUESE OLIVE (*OLEA EUROPAEA* L.) LEAF CULTIVARS

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Twenty three samples of 18 Portuguese olive leaf cultivars were analysed by a reversed-phase HPLC/DAD procedure and eight flavonoidic compounds were identified and quantified (luteolin 7,4′-*O*-diglucoside, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-rutinoside, luteolin 4′-*O*-glucoside, luteolin, apigenin and diosmetin). Luteolin 7,4′-*O*-diglucoside and luteolin 4′-*O*-glucoside were identified by HPLC/DAD/MS/MS – ESI. The studied olive leaf samples showed a common phenolic pattern, in which luteolin 4′-*O*-glucoside was almost always the major compound.

Keywords: *Olea europaea* L.; Olive leaves; Olive cultivars; HPLC DAD/MS/MS/ESI; Flavonoids

INTRODUCTION

Olea europaea L. is a typical Mediterranean tree widely cultivated for the production of olive oil and table olives. In turn the leaves found a place in popular medicine: traditionally, they have been used to treat and prevent hypertension, and for their hypoglycemic, antiseptic and diuretic properties [1–3]. They were formerly used to combat fevers, namely in malaria, but this use was dropped [2]. Tests on animals have proved its anti-hypertension and hypoglycemic properties, which many authors have attributed to the presence of oleuropein and to the chemically related oleoside [1].

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TABLE I Olive leaves characterization

Geographic origin	*Cultivar*	*Sample*
Macedo de Cavaleiros	Bical	A1
	Cordovesa	A2
	Madural	A3
	Verdeal Transmontana	A4
Mirandela	Borrenta	B1
	Redondal	B2
Mogadouro	Bical	C1
	Borreira	C2
	Madural Fina	C3
	Madural Negra	C4
	Roupuda	C5
	Santulhana	C6
Valpaços	Borrenta	D1
	Cobrançosa	D2
	Lentisca	D3
	Madural	D4
	Verdeal Transmontana	D5
Figueira de Castelo Rodrigo	Cornicabra	E1
	Negrinha do Freixo	E2
Fundão	Bical de Castelo Branco	F1
	Cobrançosa	F2
	Cordovil de Castelo Branco	F3
	Galega	F4

Spasmolytic, anti-arrhytmic, anti-septic and diuretic capacities were also proved in animal tests [2].

Chemically, olive leaves are characterized by the presence of oleuropein (that can reach up to 6–9% of dry matter) and other related seco-iridoids [3], but flavonoids, triterpenes and other classes of compounds have also been isolated [1].

Although much of the observed pharmacological activity has been attributed to the seco-iridoids, authors also reached the conclusion that other compounds must be implicated in the referred activity [3]. Flavonoids, for their many described properties, namely anti-oxidant, anti-hypertension, anti-atherogenic, anti-inflammatory, anti-allergic, anti-carcinogenic, hypoglycemic and hypocholesterolemic, anti-bacterial and anti-fungal [4,5], may also be responsible for a part of the pharmacological actions of olive leaves or, at least, for reinforcing synergistically those actions.

The literature reports several compounds of this class as occurring in olive leaves: rutin, luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside, luteolin 4′-*O*-glucoside, apigenin 7-*O*-glucoside and apigenin 7-*O*-rutinoside [6]. However, little or nothing is known about their constancy, amounts or distribution among cultivars. The purpose of this study was to identify and quantify the main flavonoids present in the methanolic extracts obtained from *O. europaea* leaves of 18 cultivars grown in Portugal (Table I), in order to evaluate the correspondent phenolic profile.

RESULTS AND DISCUSSION

Recently an HPLC/DAD method was developed for the determination of the phenolic profile of *O. europaea* L. fruits [7]. Since the literature shows that the phenolic profile of

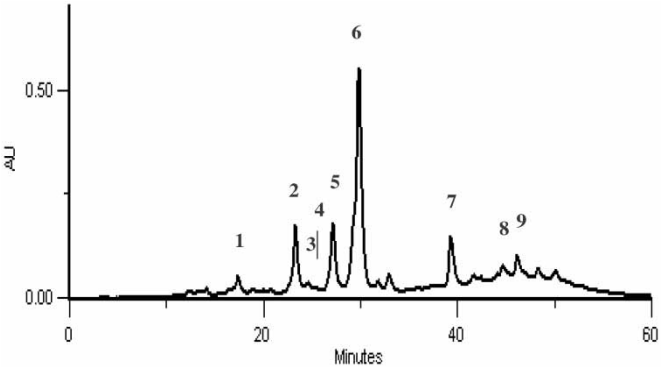

FIGURE 1 HPLC phenolic profile of a sample from Verdeal Transmontana cultivar; detection at 350 nm. (1) luteolin 7,4′-*O*-diglucoside; (2) luteolin 7-*O*-glucoside; (3) rutin; (4) apigenin 7-*O*-rutinoside (5) apigenin 7-*O*-glycoside; (6) luteolin 4′-*O*-glucoside; (7) luteolin; (8) apigenin; (9) diosmetin.

olive fruits is quite similar to that of olive leaves [8], the referred technique was now applied to the leaves.

The applied technique allowed the identification of several flavonoidic and non-flavonoidic compounds: tyrosol, caffeic acid, verbascoside, oleuropein, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-rutinoside, luteolin, apigenin and diosmetin. Figure 1 shows the chromatogram obtained with a sample from Verdeal Transmontana cultivar.

The identity of compound **6** as luteolin 4′-*O*-glucoside was based mainly on the analysis of its mass spectra: a pseudomolecular ion $[M-H]^-$ at m/z 446.5 was found for this compound, whose fragmentation provided a characteristic m/z at 285.0, a typical mass in the negative mode of the luteolin aglycon. These data confirmed the presence of a luteolin glucoside. According to its retention time and UV spectra [9] the sugar molecule should be at 4′, once the hydroxyl group at this position is more acidic than at 7 and its glycosylation would lead to an higher retention time. From the available data compound **6** was identified as luteolin 4′-*O*-glucoside.

The mass spectra of compound **1** exhibited a pseudomolecular ion $[M-H]^-$ at m/z 609.1. Its fragmentation provided a m/z at 446.5 and another m/z at 285.0, characteristic of the luteolin aglycon. The UV spectra of this compound suggested the presence of a luteolin diglucoside, with one of the sugars at position 4′. Considering these data and its retention time, compound **1** was identified as luteolin 7,4′-*O*-diglucoside. As far as we know, this is the first report of this compound in olive leaf.

All samples showed a compound with a retention time (Rt) of 27.16 min (peak **5**, Fig. 1) exhibiting a UV-vis spectra typical of an apigenin derivative. The compound presented the same Rt as a standard of apigenin 7-*O*-glucoside, already described to occur in olive leaves and fruits. However, the analysis of its mass spectra revealed that this peak corresponds to a mixture of apigenin derivatives, in which apigenin 7-*O*-glucoside is not present. These data raises some questions about the identifications previously reported. This mixture was quantified as apigenin 7-*O*-glucoside and registered in Fig. 1 and Table II as apigenin 7-*O*-glycoside.

TABLE II Flavonoidic composition of olive leaf samples (mg/kg)[a]

Sample	*Compounds*[b]									Σ
	1 *(Rt 17.42 min)*	**2** *(Rt 23.32 min)*	**3** *(Rt 24.48 min)*	**4** *(Rt 25.49 min)*	**5** *(Rt 27.16 min)*	**6** *(Rt 29.73 min)*	**7** *(Rt 38.79 min)*	**8** *(Rt 44.15 min)*	**9** *(Rt 45.71 min)*	
A1	65.6 (2.94)	386.2 (16.61)	tr	tr	324.1 (5.85)	861.9 (79.10)	167.9 (5.48)	40.5 (1.52)	92.3 (4.09)	1938.5
A2	53.7 (4.91)	586.3 (28.92)	tr	nd	437.7 (5.74)	948.2 (11.36)	581.2 (1.67)	100.1 (3.05)	194.4 (21.59)	2901.6
A3	49.9 (1.36)	771.5 (6.17)	28.4 (7.40)	tr	344.7 (4.75)	995.3 (4.84)	82.9 (1.15)	25.2 (0.59)	350.8 (0.50)	2648.7
A4	100.8 (7.58)	468.2 (10.02)	63.6 (3.65)	15.1 (3.25)	586.6 (3.62)	2013.5 (263.51)	231.5 (1.58)	97.3 (0.76)	122.1 (0.34)	3698.7
B1	tr	227.3 (4.96)	tr	tr	340.2 (0.70)	364.1 (87.50)	135.2 (0.64)	124.4 (1.22)	189.9 (2.50)	1381.1
B2	tr	211.4 (5.85)	tr	nd	342.4 (3.64)	783.2 (12.79)	338.6 (2.58)	187.4 (27.35)	204.8 (10.35)	2067.8
C1	55.4 (4.05)	282.0 (1.65)	tr	tr	359.3 (6.21)	1574.8 (19.34)	328.6 (2.51)	156.9 (1.63)	309.1 (2.82)	3066.1
C2	66.4 (5.92)	621.7 (3.83)	tr	nd	582.6 (18.31)	1476.5 (49.37)	216.1 (3.25)	103.5 (8.04)	168.6 (14.62)	3235.4
C3	121.4 (6.98)	1324.4 (30.35)	tr	tr	745.6 (35.13)	1806.4 (42.35)	277.0 (6.18)	115.7 (8.06)	40.1 (2.61)	4430.6
C4	53.1 (2.11)	1197.2 (1.48)	32.2 (2.38)	tr	496.1 (3.75)	1431.5 (11.37)	154.9 (2.01)	37.3 (0.40)	99.7 (5.50)	3502.0
C5	36.6 (6.59)	232.4 (0.60)	tr	nd	272.5 (3.50)	768.3 (3.71)	366.2 (2.01)	138.8 (0.28)	277.6 (6.16)	2092.4
C6	63.1 (2.12)	1133.3 (105.95)	tr	20.5 (1.23)	228.3 (4.30)	1632.0 (1.46)	172.1 (3.25)	126.6 (0.63)	67.9 (2.06)	3443.8
D1	tr	651.9 (32.08)	108.9 (1.03)	nd	333.6 (2.96)	846.4 (77.81)	113.8 (2.62)	51.6 (3.95)	105.4 (3.10)	2211.6
D2	103.0 (11.74)	585.1 (19.60)	308.9 (35.09)	tr	404.9 (12.58)	815.3 (19.60)	68.5 (2.09)	4.6 (0.59)	75.5 (2.22)	2365.8
D3	76.9 (2.42)	976.8 (29.38)	290.8 (38.42)	tr	373.5 (8.44)	1536.5 (66.32)	162.8 (7.59)	121.9 (0.82)	46.5 (0.50)	3585.7
D4	104.9 (33.91)	1954.5 (113.77)	116.5 (9.03)	tr	550.9 (2.93)	1750.5 (13.98)	93.2 (1.20)	9.4 (0.25)	18.9 (7.35)	4598.8
D5	7.8 (0.06)	171.2 (0.27)	tr	tr	122.7 (1.33)	253.1 (0.80)	92.5 (0.30)	27.5 (0.56)	122.0 (0.88)	796.8
E1	116.6 (2.98)	1614.8 (60.11)	292.7 (6.26)	60.3 (1.99)	818.2 (33.10)	2673.8 (32.11)	689.8 (43.88)	222.8 (50.64)	tr	6489.0
E2	119.4 (9.04)	591.1 (11.35)	125.1 (3.08)	nd	433.9 (10.94)	1979.4 (98.90)	778.8 (11.46)	113.1 (7.57)	245.9 (2.23)	4386.7
F1	7.4 (0.05)	85.2 (0.13)	tr	nd	218.9 (12.07)	312.3 (27.53)	312.8 (6.21)	103.5 (0.07)	266.8 (1.42)	1306.9
F2	47.8 (2.85)	181.8 (6.30)	tr	tr	491.0 (14.12)	879.0 (56.90)	44.7 (1.74)	75.3 (0.61)	157.5 (8.36)	1877.1
F3	53.0 (2.01)	718.9 (8.50)	44.2 (1.62)	tr	276.9 (1.94)	1106.3 (3.10)	169.2 (4.06)	32.7 (7.66)	119.9 (17.09)	2521.1
F4	tr	866.2 (0.56)	tr	tr	1261.3 (2.87)	1167.2 (44.36)	198.1 (5.03)	339.5 (6.77)	116.4 (3.25)	3948.7
Range	0.0–121.4	85.2–1954.5	tr – 308.9	nd – 60.3	122.7–1261.3	253.1–2673.8	44.7–778.8	4.6–339.5	tr – 350.8	
Mean	56.6	688.7	141.1	32.0	449.8	1216.3	251.1	102.3	147.4	

[a] Values are expressed as mean (standard derivation) of three assays for each sample; tr, traces; nd, not detected; Σ, sum of the determined compounds. [b] Identity of compounds as in Fig. 1.

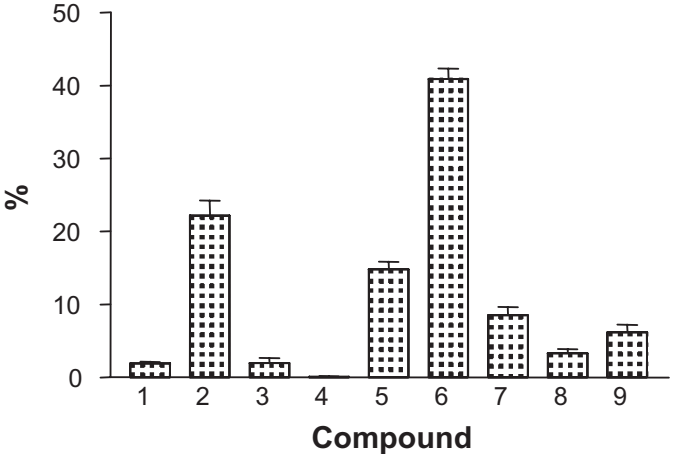

FIGURE 2 Flavonoidic fingerprint of Bical, Cordovesa, Madural, Verdeal Transmontana, Borrenta, Redondal, Borreira, Madural Fina, Madural Negra, Roupuda, Santulhana, Cobrançosa, Lentisca, Cornicabra, Negrinha do Freixo and Cordovil de Castelo Branco cultivars. Identity of compounds as in Fig. 1.

The compounds tyrosol, cafeic acid and verbascoside were present in most of the samples, but in some of them they were present only in trace amounts. Only the flavonoidic compounds were quantified and the results are shown in Table II.

On comparing the obtained results with those published in literature, some remarks can be made. Luteolin-7-*O*-rutinoside was not found in any of the analysed samples. Rutin and apigenin 7-*O*-rutinoside, although also described in olive leaves, were not present in all samples and, when detected, they were found in small amounts. This means that these compounds should not be considered characteristic of the flavonoidic profile of olive leaves and, therefore, not used for the quality control. On the other hand, all other identified flavonoids seemed to be reasonably constant. The quantitative profile revealed many similarities. Luteolin 4′-glucoside is always the major compound (ranging from 24–54% of total flavonoids), with the exception of the samples from Galega (sample F4) and Bical de Castelo Branco (sample F1) cultivars. The second or third peaks are almost always those identified as luteolin 7-*O*-glucoside and apigenin 7-*O*-glycoside. On analyzing the data obtained in terms of percentage, the resultant chemical fingerprint (Fig. 2) can relate all cultivars except those already mentioned (Galega and Bical de Castelo Branco).

In some cases, samples from two geographical origins for each cultivar were analysed (pairs A1/C1, A3/D4, A4/D5, B1/D1 and D2/F2). The charts obtained are not superimposable (data not shown) and this is not surprising since many factors, specially the climate and the nature of the soil, are known to influence the metabolism of the compounds.

This preliminary study of phenolic compounds of olive leaves from cultivars grown in Portugal does not seem to indicate marked differences in their phenolic profiles, according to the cultivar. However, more studies are needed involving more cultivars and from several origins, in order to establish a chemical fingerprint that can be useful in demonstrating the sameness or difference among herbal medicines containing olive leaves.

EXPERIMENTAL

Standards and Reagents

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Samples

Experiments were carried out on 23 olive leaves samples of different geographic origin from 18 Portuguese cultivars (Table I). Fresh olive leaves were harvested in November 2000 in the North (Macedo de Cavaleiros, Mirandela, Valpaços, Mogadouro and Figueira de Castelo Rodrigo) and Center of Portugal (Fundão). For each sample, about 20 g of leaves were manually collected around three selected olive trees, dried at room temperature and, afterwards, stored in paper bags to protect them from light. Each sample was powdered at maximum particle size of 1600 μm just before phenolics extraction.

Extraction of Phenolic Compounds

0.2 g of each powdered sample was thoroughly mixed with 40 mL methanol (60%) for 30 min, followed by another extraction with 40 mL of methanol (60%), for 10 min, at 45°C. The hidromethanolic extracts were gathered, filtered, evaporated to dryness under reduced pressure (40°C), redissolved in methanol (1 mL), and 20 μL were analysed by HPLC.

HPLC/DAD Analysis

Chromatographic separation was carried out with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (250 × 4.6 mm, 5 μm particle size, Merck, Darmstadt, Germany) column. The solvent system used was a gradient of water/formic acid (19 : 1) (A) and methanol (B), starting with 15% methanol and installing a gradient to obtain 18% B at 3 min, 25% B at 6 min, 39% B at 19 min, 39% B at 22 min, 50% B at 36 min, 79% B at 59 min and 100% B at 60 min. The flow rate was 0.9 mL/min. Detection was accomplished with a diode array detector, and chromatograms were recorded at 280, 320 and 350 nm.

Spectral data from all peaks were accumulated in the range 200–400 nm. The data were processed on a Unipoint® system software (Gilson Medical Electronics, Villiers le Bel, France). The identification of tyrosol, caffeic acid, verbascoside, oleuropein, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-rutinoside, luteolin, apigenin and diosmetin was made by comparison of their retention times and UV-vis spectra with those obtained from standards. These identifications were confirmed by HPLC/DAD/MS/MS.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, with detection at 280 nm for tyrosol, at 320 nm for caffeic acid and verbascoside and at 350 nm for all flavonoids. Verbascoside

was quantified as caffeic acid and luteolin 4′-*O*-glucoside as luteolin 7-*O*-glucoside. The other compounds were quantified as themselves.

HPLC/DAD/MS/MS Analysis

The analysis was performed following the chromatographic conditions referred above. The HPLC system was equipped with a DAD and mass detector in series (Agilent 1100 Series LC/MSD Trap). It consisted of an Agilent G1312A HPLC binary pump, an Agilent G1313A autosampler, an Agilent G1322A degasser and an Agilent G1315B photo-diode array detector controlled by Agilent software v. A.08.03 (Agilent Technologies, Waldbronn, Germany). The mass detector was an Agilent G2445A ion-trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionisation (ESI) system and controlled by Agilent Software v. 4.0.25. Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted at 11 L/min. The heated capillary and voltage were maintained at 350°C and 4 kV, respectively. The full scan mass spectra of the phenolic compounds were measured from m/z 100 up to m/z 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, the collision energy was set at 100%. Mass spectrometry data were acquired in the negative ionisation mode.

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