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PHENOLIC PROFILE OF HAZELNUT (*Corylus avellana* L.) LEAVES CULTIVARS GROWN IN PORTUGAL

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In this study, phenolic compounds of hazelnut leaves of 10 different cultivars with the same cultural, geographical, geological and climatic conditions were analyzed by HPLC/DAD and HPLC/DAD/MS/MS – ESI. Eight phenolic compounds (3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeoyltartaric acid, *p*-coumaroyltartaric acid, myricetin 3-rhamnoside, quercetin 3-glycoside, quercetin 3-rhamnoside and kaempferol 3-rhamnoside) were identified and quantified. All of the analyzed samples showed a similar phenolic profile, in which myricetin 3-rhamnoside and quercetin 3-rhamnoside were the major compounds and caffeoyltartaric and *p*-coumaroyltartaric acids were present in vestigial amounts.

Keywords: Corylus avellana L.; Hazelnut leaves; Phenolic compounds; HPLC/DAD; HPLC/DAD/MS/MS – ESI

INTRODUCTION

The study of bioactive phytochemicals found in many plants and plant foods, and their possible healthy effects, represents an area of growing interest in the fields of nutritional and biological chemistry. Antioxidants from plants, namely phenolic compounds [1–3], can be used in order to preserve food, for organoleptic and nutritional qualities, and also for medicinal purposes [4,5].

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Hazelnut (*Corylus avellana* L.) has a wide geographical distribution in Europe, comprising a large range of climates, although for economical reasons production is limited to well defined environmental conditions, characterized by mild, humid winters and dry summers [6]. Hazelnut leaves have been used in folk medicine in varicose veins and haemorrhoidal symptomatology, as a result of their vasoconstrictor and antihaemorrhagic properties. Slight antidysenteric, antifungical and cicatrizant properties have also been described [7,8].

However, as far as we know, few studies have been published on hazelnut leaves and only the major phenolic compounds (5-caffeoylquinic acid, myricetin 3-rhamnoside, quercetin 3-rhamnoside and kaempferol 3-rhamnoside) have been reported [7,9]. The purpose of this study is to contribute to the characterization of the phenolic fraction of hazelnut leaves, since it may be related to the therapeutic effects of this herbal drug. Phenolic compounds of hazelnut leaves obtained from different cultivars, with the same cultural, geographical, geological and climatic conditions were identified by HPLC/DAD/MS/MS – ESI and quantitatively analyzed by HPLC/DAD.

RESULTS AND DISCUSSION

The UV spectra of the compounds obtained by HPLC/DAD analysis revealed that phenolic acids and flavonoids were the two main groups of compounds in hazelnut leaf extract. The first case corresponds to peaks 1, 2, 3 and 4 (Fig. 1). The comparison of the retention time and UV spectra (245, 290 sh, 325 nm) of compounds **1** and **2** with those of standards suggests the presence of cinnamic acid derivatives. Data from the MS/MS study of both compounds is identical: fragmentation of pseudomolecular ion $[M-H]^-$ at m/z 353.6 yields the ion at m/z 191.5 ($[M-H]^- - 162$), base peak, corresponding to quinic acid by the loss of a caffeoyl radical from pseudomolecular ion.

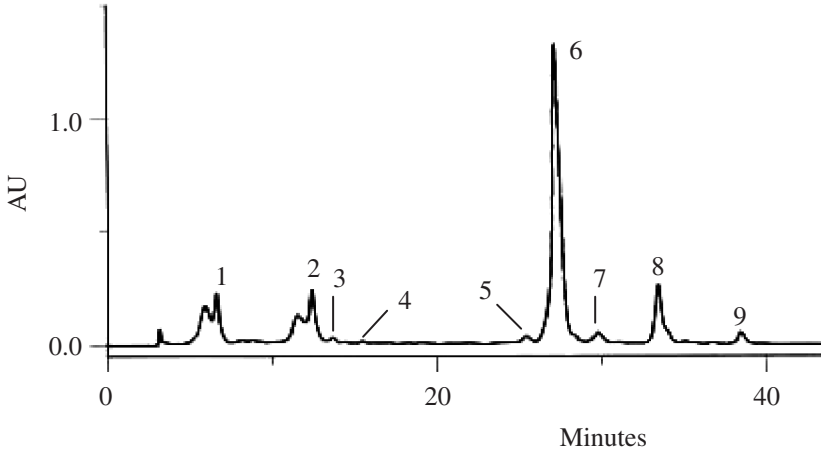

FIGURE 1 HPLC/DAD hazelnut leaf phenolic profile. Detection at 320 nm. 1: 3-caffeoylquinic acid; 2: 5-caffeoylquinic acid; 3: caffeoyltartaric acid; 4: *p*-coumaroyltartaric acid; 5: myricetin 3-hexoside + myricetin derivative; 6: myricetin 3-rhamnoside; 7: quercetin 3-hexoside + myricetin derivative; 8: quercetin 3-rhamnoside; 9: kaempferol 3-rhamnoside.

Besides, according to Cliford *et al*. [10], in the MS/MS study the ion at *m*/*z* 179.5 was also obtained with an abundance of 2% for compound corresponding to peak **2**, which characterizes 5-caffeoylquinic acid, and with an abundance of 35% for compound corresponding to peak **1**, which characterizes 3-caffeoylquinic acid. So, compound **1** was identified as 3-caffeoylquinic acid and compound **2** was identified as 5-caffeoylquinic acid. HPLC-MS analysis of compound **3** showed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 311.4. The MS/MS of $[M-H]^-$ ion yielded two ions at *m*/*z* 179.2 ($[M-2H]^- - 132$) and 149.2 ($[M-H]^- - 162$) corresponding respectively to the loss of tartaric acid and caffeic acid. According to these data, compound **3** was identified as caffeoyltartaric acid. Compounds **3** and **4** have identical MS/MS patterns profiles differing in the fact that ions from compound **4** have an *m*/*z* 16 u.m.a lower than those from compound **3**. So, fragmentation of pseudomolecular ion $[M-H]^-$ at *m*/*z* 295.3 yielded an ion at *m*/*z* 163.1, which corresponds to *p*-coumaric acid – H and compound **4** was identified as *p*-coumaroyltartaric acid.

The second group of compounds (peaks **5**–**9**) presents UV spectra characteristic of flavonoids. For peak **5** data suggest that there are two compounds coeluting (**5a**, **5b**). One of them (**5a**) probably is a myricetin 3-hexoside, since the MS/MS study of its pseudomolecular ion $[M-H]^-$ at *m*/*z* 479.1 provided a characteristic *m*/*z* at 316.0, a typical mass in the negative mode of the myricetin aglycon (318 − 2 H). The other compound (5b) showed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 625.2 that yielded 3 ions in the MS/MS study: 478.9 ($[M-H]^-$ – Rhamnose), 462.9 ($[M-H]^-$ – Glucose) and 317.0 [479–162 (Hexose) and 463 − 146 (Rhamnose)]. These data suggest the presence of myricetin(317) + hexose(162) + rhamnose(146). The analysis of UV spectra of this compound suggests that hydroxyl group in position 3 is not free. The two sugar molecules are probably linked to different phenolic hydroxyl groups, since the MS/MS shows that the ions resulting from loss of one sugar are more important than those of the aglycon, in opposition to what is verified with rhamnoglucosides, in which the base peak ion is that of the aglycon. Since the main flavonoid present in all analyzed samples is myricetin 3-rhamnoside, and considering the biosynthetic pathway, rhamnose is probably the sugar in position 3 and the hexose may be in position 7 or in ring B (position 3′ or 4′). In order to get more information about this compound we have performed an HPLC-DAD/MS/MS study of two isomers differing in the position of the sugar: isorhamnetin 7-glucoside and isorhamnetin 4′-glucoside. The data obtained from the elution order suggest that compound **5b** is possibly myricetin 3-rhamnoside-7-hexoside.

A pseudomolecular ion $[M-H]^-$ at *m*/*z* 463.2 was found for peak **6**. Fragmentation of this ion provided a characteristic *m*/*z* at 316.1 ($[M-H]^-$ – Rhamnose), a typical mass in the negative mode for myricetin aglicone. Comparison of retention times, UV-Vis spectra and MS data with those obtained from an authentic standard of myricetin 3-rhamnoside confirmed the occurrence of this compound in hazelnut leaf.

In peak **7** there are two compounds coeluting with pseudomolecular ions $[M-H]^-$ at *m*/*z* 463.6 and 625.2. One of them (**7a**) is probably a quercetin 3-hexoside since the MS/MS study of its pseudomolecular ion $[M-H]^-$ at *m*/*z* 463.6 provides a characteristic *m*/*z* at 300.8, a typical mass in the negative mode of the quercetin aglycon. The other compound (**7b**) probably is an isomer of compound **5b** since their MS/MS indicate identical patterns.

For peak **8** was obtained a pseudomolecular ion $[M-H]^-$ at *m*/*z* 447.3. Fragmentation of this ion yielded a characteristic *m*/*z* at 300.9 ($[M-H]^-$ – Rhamnose), a typical mass in

the negative mode for quercetin aglicone. Comparison of analytical data with those obtained from an authentic standard allowed us to confirm the presence of quercetin 3-rhamnoside in hazelnut leaf.

Compound **9** gave a pseudomolecular ion $[M-H]^-$ at m/z 431.3. Fragmentation of this ion provided a characteristic m/z at 285.0 ($[M-H]^-$ – Rhamnose), a typical mass in the negative mode for a tetrahydroxyflavone, which was identified as kaempferol according to its UV spectra. For that reason, compound **9** must be kaempferol 3-rhamnoside.

In general terms, all samples presented a common qualitative compositional pattern, composed by seven identified phenolic compounds: 5-caffeoylquinic acid, caffeoyltartaric acid, *p*-coumaroyltartaric acid, myricetin 3-rhamnoside, quercetin 3-rhamnoside, quercetin 3-glycoside and kaempferol 3-rhamnoside. Differences among cultivars were only found for two compounds: 3-caffeoylquinic acid (present in only four cultivars) and caffeoyltartaric acid (not present in one cultivar).

Considering that for the determination of the chemical identity of a given species, not only the qualitative characterization of its constituents is important but also the knowledge of their amounts and ratios, the compounds of hazelnut leaves were quantified (Table I). All the analyzed samples exhibited a common quantitative pattern, in which myricetin 3-rhamnoside was the major compound, followed by quercetin 3-rhamnoside, in similar amounts to those already reported by Fraisse *et al.* [7].

From this preliminary study of the phenolic compounds of hazelnut leaves, it seems that the nature of the cultivar does not influence the phenolic profile of this matrix. However, it would be interesting to study the influence of other factors, such as the cultural practices and geographical origin.

EXPERIMENTAL

Standards and Reagents

The standards were purchased from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). 3-*O*-Caffeoylquinic acid was not commercially available, so it was prepared by transesterification of 5-*O*-caffeoylquinic acid (chlorogenic acid) [11,12]. 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

Studies were carried out on hazelnut leaves of 10 cultivars grown in Portugal. An experimental plantation was established in March 1984, in Vila Real, north-eastern Portugal (41° 19′ N, 7° 44′ W, 470 m a.s.l.). Trees were planted at 5×3 m spacing with no pruning, in a complete randomized plot design for fruit sampling. All of the fresh samples were collected on the same day, in July of 2002. For each sample, about 20 g of leaves were manually collected from the middle third of branches exposed to sunlight, dried in a stove for two days at 30°C and stored in paper bags in order to protect them from light. Immediately before the phenolic extraction, each sample was powdered at a maximum particle size of 910 μm.

TABLE I Phenolic composition of hazelnut leaf samples (g/Kg, dry basis)[a]

	Compounds									
Cultivar	**1**	**2**	**3**	**4**	**5a + 5b**	**6**	**7a + 7b**	**8**	**9**	Σ
Butler	nd	2.34 (0.03)	0.02 (0.00)	0.02 (0.00)	0.92 (0.05)	18.24 (0.62)	1.01 (0.08)	3.56 (0.03)	0.37 (0.01)	26.48
Campaniea	nd	1.69 (0.04)	0.02 (0.00)	0.01 (0.00)	1.29 (0.01)	14.01 (0.30)	1.44 (0.03)	3.68 (0.03)	0.28 (0.01)	22.42
Ennis	nd	3.34 (0.07)	0.01 (0.00)	0.02 (0.00)	1.36 (0.00)	18.07 (0.47)	1.15 (0.02)	3.44 (0.01)	0.30 (0.01)	27.68
F. Coutard	nd	1.93 (0.00)	0.01 (0.00)	0.01 (0.00)	0.44 (0.01)	14.79 (0.05)	1.14 (0.01)	3.53 (0.02)	0.35 (0.00)	22.19
Grossal	nd	1.21 (0.02)	0.01 (0.00)	0.01 (0.00)	0.53 (0.02)	10.60 (0.00)	0.58 (0.01)	2.20 (0.00)	0.27 (0.00)	15.41
Lansing	0.07 (0.00)	2.33 (0.01)	0.01 (0.00)	0.02 (0.00)	0.90 (0.01)	13.71 (0.08)	0.54 (0.01)	2.09 (0.03)	0.32 (0.00)	19.98
M. Bollwiller	1.68 (0.00)	0.93 (0.03)	nd	0.01 (0.00)	0.49 (0.01)	12.66 (0.00)	1.14 (0.02)	4.50 (0.06)	0.25 (0.00)	21.67
Segorbe	1.43 (0.01)	1.13 (0.03)	0.01 (0.00)	0.01 (0.00)	0.48 (0.01)	16.36 (0.06)	0.23 (0.00)	1.57 (0.04)	0.19 (0.00)	21.40
St. M[a] Jesus	nd	1.34 (0.02)	0.01 (0.00)	0.01 (0.00)	0.47 (0.01)	14.43 (0.25)	0.84 (0.02)	4.68 (0.13)	0.39 (0.01)	22.16
Tonda Giffoni	1.16 (0.00)	1.11 (0.00)	0.02 (0.00)	0.00 (0.00)	0.30 (0.01)	11.71 (0.01)	0.33 (0.01)	1.74 (0.00)	0.22 (0.01)	16.59

[a]Values are expressed as mean (standard deviation) of three determinations for each sample. nd: not detected; Σ: sum of the determined compounds; 1: 3-caffeoylquinic acid; 2: 5-caffeoylquinic acid; 3: caffeoyltartaric acid; 4: *p*-coumaroyltartaric acid; 5a + 5b: myricetin 3-hexoside + myricetin derivative; 6: myricetin 3-rhamnoside; 7a + 7b: quercetin 3-hexoside + myricetin derivative; 8: quercetin 3-rhamnoside; 9: kaempferol 3-rhamnoside.

Extraction of Phenolic Compounds

Each sample (*ca*. 0.15 g) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to NaOH 20%). The methanolic extract was filtered, evaporated to dryness under reduced pressure (40°C) and redissolved in methanol (1 mL).

HPLC/DAD Analysis

Chromatographic separation was achieved 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 30% B at 15 min, 45% B at 30 min, 52.5% B at 40 min, and 100% B at 41 min. The flow rate was 1 mL/min, and the injection volume was 20 µl. Detection was accomplished with a diode array detector (Gilson), and chromatograms were recorded at 320 and 350 nm.

Spectral data from all peaks were accumulated in the 200–400 nm range. Data were processed on a Unipoint® system software (Gilson Medical Electronics, Villiers le Bel, France).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, with detection at 320 nm for phenolic acids and at 350 nm for flavonoids. 3-*O*-Caffeoylquinic acid was quantified as 5-*O*-caffeoylquinic acid, caffeoyltartaric acid was quantified as caffeic acid, *p*-coumaroyltartaric acid was quantified as *p*-coumaric acid, the mixture of myricetin derivatives (on peak 5) was quantified as myricetin 3-rhamnoside, the mixture on peak 7 was quantified as quercetin 3-glycoside, and kaempferol 3-rhamnoside was quantified as kaempferol 3-glucoside. The other compounds were quantified as themselves.

HPLC/DAD/MS/MS System for Qualitative Analysis

Chromatographic separation was carried out on a LiChroCART column (250 × 4 mm, RP-18, 5 µm particle size, Merck, Darmstadt, Germany) using two solvents: trifluoracetic acid (0.1%) (A) and methanol (B), starting with 30% methanol and installing a gradient to obtain 50% B at 30 min and 70% B at 32 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Detection was carried out at 280, 320 and 350 nm.

The HPLC system was equipped with a DAD and mass detector in series (Agilent 1100 Series LC/MSD Trap). It consisted of an Agilent G1312 A HPLC binary pump, an Agilent G1313 A autosampler, an Agilent G1322 A 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 G2445 A 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 60 up to m/z 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with a voltage ramping to 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MS/MS data were acquired in the automatic mode.

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