PHYTOCHEMICAL ANALYSIS
*Phytochem. Anal.* **16**, 380–387 (2005)
Published online 11 August 2005 in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002.pca.864

# Characterisation of Polyphenols by HPLC-PAD-ESI/MS and Antioxidant Activity in *Equisetum telmateia*

**Helena Correia,[1] Ana González-Paramás,[2] Maria Teresa Amaral,[1] Celestino Santos-Buelga[2] and Maria Teresa Batista[1]***

[1]Centro de Estudos Farmacêuticos and Laboratório de Farmacognosia, Faculdade de Farmácia Universidade de Coimbra, 3000-295 Coimbra, Portugal
[2]Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, 37007 Salamanca, Spain

**The antioxidant activity of an aqueous extract (infusion) and respective ethyl acetate fraction of *Equisetum telmateia* Ehrh. (Equisetaceae), a plant used in traditional medicine for its anti-inflammatory and diuretic properties, has been evaluated by DPPH, TEAC and TBARS assays. A high and significant antioxidant activity was detected in the ethyl acetate fraction. Analysis of the aqueous extract and the ethyl acetate fraction by HPLC-PAD-ESI/MS allowed the identification of the major phenolic compounds as flavan-3-ol, kaempferol and phenolic acid derivatives. Among the flavan-3-ols, A-type proanthocyanidins and afzelechin derivatives were detected as well as the more common B-type procyanidins, $B_2$ and $C_1$, whose identification was further confirmed by HPLC using detection involving chemical reaction with *p*-dimethylamino-cinnamaldehyde. The results suggest that the anti-inflammatory activity of *E. telmateia* could be due, at least in part, to the presence of compounds with antioxidant activity. Copyright © 2005 John Wiley & Sons, Ltd.**

*Keywords:* HPLC; mass spectroscopy; flavonoids; proanthocyanidins; afzelechins; kaempferol derivatives; antioxidant activity; *Equisetum telmateia*; Equisetaceae.

## INTRODUCTION

*Equisetum telmateia* Ehrh. (Equisetaceae) is a species of the subgenus *Equisetum* and is widely distributed in Europe. The aerial parts of this plant have been used for the treatment of prostatitis, stomach aches and cystitis in traditional medicine (Tuzlaci and Tolon, 2000, 2001). Veit *et al*. (1995) documented the predominance of kaempferol derivatives, especially acetylated glycosides in *E. telmateia* and showed the exclusiveness of such acetylated glycosides in this species. However, to our knowledge, no information is available so far concerning the bioactivity of *E. telmateia*.

Recent studies confirm the beneficial effects of kaempferol and its derivatives in protection against various pathological states such as inflammation, arteriosclerosis, cancers and viral diseases (Liang *et al*., 1999; Min *et al*., 2001; Ho *et al*., 2002; Huss *et al*., 2002; Olszaneicki *et al*., 2002). Similar effects have been reported for other classes of flavonoids such as the flavanols (i.e. catechins and proanthocyanidins; Santos-Buelga and Scalbert, 2000). The physiological mechanisms involved in the health-fortifying effects of the flavonoids are still unclear owing to the lack of information about their bioavailability; however, *in vitro* studies suggest that antioxidant mechanisms are involved in most of these effects.

* Correspondence to: M. T. Batista, Centro de Estudos Farmacêuticos and Laboratório de Farmacognosia, Faculdade de Farmácia Universidade de Coimbra, 3000-295 Coimbra, Portugal.
Email: mtpmb@ci.uc.pt
Contract/grant sponsor: FCT.
Contract/grant sponsor: Programa Operacional de Ciência, Tecnologia e Inovação.

Flavonoids interact with mammalian enzyme systems, including nitric oxide synthase (NOS), which is found in two isoforms, constitutive (cNOS) and inductive (iNOS). The NO produced by the cNOS isoform acts as a vasodilator and inhibits leukocyte and platelet aggregation. The iNOS is induced by inflammatory stimuli and produces NO in high quantities: under these conditions, NO promptly reacts with superoxide anion producing peroxynitrite. Some studies have shown that certain flavonoids could inhibit NO production, probably by inhibition of iNOS and, conversely, stimulate cNOS activity (Karim *et al*., 2000; Olszaneicki *et al*., 2002) acting as antioxidants in the inflammatory processes.

Other antioxidant mechanisms deal with the scavenging of reactive oxygen and nitrogen species (ROS and RNS, respectively), reducing in this way the oxidative stress responsible for cell damage. Moreover, the scavenging of peroxyl radicals can reduce the extent of lipid peroxidation and low-density lipoprotein (LDL) oxidation and, consequently, the formation of atherosclerotic plaques. Furthermore, it is known that oxidative stress, lipid peroxidation and LDL oxidation are also associated with some cancers, inflammation and coronary heart diseases (Middleton *et al.*, 2000). Flavonoids can also interact with transition metal ions such as iron and copper to form chelates and thereby probably reduce their reactivity (Rice-Evans, 2001).

Since it is possible that the pharmacological properties of *E. telmateia* Ehrh. could be due at least in part to its flavonoid content, the polyphenolic composition of an infusion (and its ethyl acetate fraction) of the plant has been analysed with the aim of evaluating the antioxidant activity and identifying the components responsible for such activity.

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*Received 5 March 2004*
*Revised 7 June 2004*
*Accepted 14 June 2004*

## EXPERIMENTAL

### Reference standards

Kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were purchased from Extrasynthese (Genay, France). Standards of flavan-3-ols were obtained from grape seeds by repeated extraction with methanol and fractionation of the combined extracts on a Sephadex LH-20 Fluka (Buchs, Switzerland) column (45 × 5 cm i.d.) using ethanol as mobile phase. Catechins and proanthocyanidins were further separated from the Sephadex fractions by semi-preparative HPLC as described by de Pascual-Teresa *et al.* (1998a).

### Plant material and preparation of extracts

Dried aerial parts of *Equisetum telmateia* Ehrh. were provided by Segredo da Planta, Portugal. Plant material was identified by Dr. J. Paiva (Botany Department, University of Coimbra, Portugal), and a voucher has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Coimbra. Extracts were prepared from the pulverised plant material according to their uses in traditional medicine.

An infusion was prepared by adding 100 mL of hot water to 5 g of the plant material. The mixture was kept hot and left to stand for 10 min, following which the mixture was filtered under vacuum and the filtrate cooled and its volume made up to 100 mL with water. An aliquot of the infusion was washed with *n*-hexane and further extracted with ethyl acetate. The organic phase was collected, dried over anhydrous sodium sulphate and, after the addition of water concentrated under vacuum at 30°C until all of the ethyl acetate had been removed. The infusion and the ethyl acetate fraction were frozen with liquid nitrogen and then lyophilised.

A decoction was prepared by adding 100 mL of cold water to 5 g of the plant material and heating the mixture to boiling for 20 min. After cooling, the mixture was filtered under vacuum and its volume made up to 75 mL with water.

In order to prepare a tincture, 40 mL of 45% aqueous ethanol were added to 2.5 g of the plant material and the mixture allowed to stand at room temperature for 13 days in the dark. After this time, the hydro-alcoholic extract was filtered, concentrated under vacuum and its volume made up to 10 mL with 45% aqueous ethanol.

### Determination of antioxidant activity

**DPPH assay.** An adaptation of the method described by Blois (1958) was employed in which aliquots (100 μL) of aqueous samples of different concentrations were added to 500 μL of an ethanolic solution (500 μM) of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. After mixing for 30 s, the reaction mixture was incubated in the dark at room temperature for 30 min and the absorbance measured at 517 nm on an Hitachi model U-2000 (Tokyo, Japan) spectrophotometer. The reducing activity of the sample was estimated from the decrease in absorbance (i.e. absorbance of the control-absorbance of the sample), and the results expressed as $EC_{50}$ values [defined as the amount (mg) of dry sample required to decrease by 50% the initial absorbance at 517 nm of the DPPH radical solution]. The $EC_{50}$ of an ascorbic acid standard was determined under the same assay conditions.

**TEAC assay.** The method of Miller *et al.* (1993) was employed to determine scavenging activity against 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. Aliquots (8.4 μL) of samples were mixed with 30 μL of ABTS radical (5 mM) and the absorbance of the reaction mixture was monitored at 734 nm for 6 min using a Hewlett Packard model 8453 (Waldbroan, Germany) spectrophotometer. The results were compared with those obtained using Trolox C (a water-soluble analogue of vitamin E) as a reference standard and expressed as Trolox equivalent antioxidant capacity (TEAC)/mg dry weight of sample.

**TBARS assay.** The levels of thiobarbituric acid reactive substances (TBARS) were measured using the method of Yagi (1976). Aliquots (4 μL) of samples were added to an ascorbate/iron-induced peroxidation solution of phosphatidylcholine (final concentration 1.25 mg/mL) and the resulting mixture added to 800 μL TBA solution containing 20% trichloroacetic acid (w/v) and 0.4% thiobarbituric acid (w/v) in hydrochloric acid (0.25 M). The absorbance at 535 nm was determined using a Perkin-Elmer (Norwalk, CT, USA) model Lambda 3B UV–vis spectrophotometer and the results were expressed as $IP_{50}$ values [defined as the amount (mg) of dry sample required to inhibit 50% of the induced lipid peroxidation].

### Total phenols

The total phenol content was determined using Folin–Ciocalteu's reagent according to the method described by Wang *et al.* (1997). The results were expressed in terms of g gallic acid equivalent/100 g (dry weight) of sample.

### Total flavonols

The total flavonol content was determined according to the method previously described by Lamaison and Carnat (1990). Samples in ethanol (2 mL) were mixed with 2 mL reagent containing 2% aluminium chloride hexahydrate in methanol (w/v), incubated for 10 min at room temperature in the dark, and the absorbance of the mixture measured at 430 nm in a Hitachi model U-2000 spectrophotometer. The results were expressed in terms of g rutin equivalent/100 g (dry weight) of sample.

### Total flavanols

The total flavan-3-ol content was determined by the vanillin-hydrochloric acid assay described by Julkunen-Titto (1985). Aliquots (100 μL) of samples in anhydrous methanol were mixed with 3 mL 4% vanillin solution for 1 min, after which time a volume (1.5 mL) of hydrochloric acid was immediately added. After incubation of the reaction mixture in the dark at room temperature for 20 min, the absorbance was measured at 500 nm using a Hitachi model U-2000 spectrophotometer. The results

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were expressed in terms of g catechin equivalent/100 g (dry weight) of sample.

### Analysis of the polyphenol composition by HPLC-PAD-ESI/MS

Analyses were performed using a Hewlett Packard model HP 1100 system with an autoinjector and interfaced with a Finnigan LCQ (San Jose, CA, USA) spectrometer equipped with an API/ES ionisation chamber. Separation was performed on a Waters Spherisorb ODS2 column (150 × 4.6 mm i.d.; 3 μm) maintained at 25°C. The mobile phase consisted of 2.5% acetic acid (solvent A), a 90:10 mixture of 2.5% acetic acid and acetonitrile (solvent B) and acetonitrile (solvent C). The gradient profile used was from 100:0:0 (A:B:C) to 0:100:0 between 0 and 5 min, changing to 0:85:15 between 5 and 30 min, changing to 0:50:50 between 30 and 35 min, and followed finally by isocratic elution with 0:50:50 between 35 and 40 min; the flow-rate was 0.5 mL/min. The first detection was by Photodiode Array Detector (PAD) with the spectrophotometer set at 280 and 360 nm, and the second detection employed an electrospray ionisation (ESI) MS. The capillary temperature was 225°C and the capillary voltage was 45 V. Spectra were obtained in the positive ion mode and the MS was programmed to perform two scans, a full mass scan and an $MS^2$ scan of the most abundant ion using a collision energy of 45 V.

### Analysis of flavan-3-ols by HPLC with detection following post-column derivatisation with *p*-dimethylamino-cinnamaldehyde (DMACA)

The method followed was that described by de Pascual-Teresa *et al.* (1998b). A Waters 600E HPLC system, coupled to a Waters model 717 Plus auto-sampler and a Waters Spherisorb ODS2 column (150 × 4.6 mm i.d.; 3 μm) operating at 25°C, was employed. The elution was performed with water (solvent A), methanol (solvent B), 4.5% aqueous formic acid (solvent C) and a 90:10 mixture of 4.5% aqueous formic acid and methanol (solvent D). The gradient profile used was from 100:0:0:0 (A:B:C:D) to 0:0:100:0 between 0 and 10 min, changing to 0:0:85:15 between 10 and 20 min, followed by isocratic elution with 0:0:85:15 between 20 and 30 min, changing to 0:0:65:35 between 30 and 40 min, followed by isocratic elution with 0:0:65:35 between 40 and 45 min, changing to 0:0:55:45 between 45 and 60 min, changing to 0:0:0:100 between 60 and 75 min, changing to 0:50:0:50 between 75 and 175 min, and finally changing to 0:80:0:20 between 175 and 180 min; the flow rate was 0.5 mL/min. A Hewlett-Packard model HP 1040 M PAD was used for the first detection at 280 nm, after which reagent containing 1% DMACA in 1.5 M sulphuric acid in methanol was added to the effluent flow by means of a T-connection and Kontron (Milan, Italy) model 320 pump operating at a flow rate of 0.5 mL/min. Post-column derivatisation proceeded for 2.5 min in a 9 m knitted Teflon tube (0.5 mm i.d.), after which the product of the reaction was detected at 640 nm using Shimadzu (Tokyo, Japan) model SPD-10AV UV–vis detector. Data processing was performed with a Hewlett Packard data treatment station.

## RESULTS AND DISCUSSION

### Screening with the DPPH assay

The simplicity, speed and sensitivity of the DPPH assay allows the screening of many samples in a short time, and also permits the detection of radical scavengers even when present in small concentrations (Yokozawa *et al*., 1998). Initially, various extracts of *E. telmateia* Ehrh. were screened for their antioxidant activity using this assay in order to select the extract that presented the strongest effect. Whilst all of the extracts exhibited scavenger activity, the infusion was found to be the most active, showing a scavenger activity that was ca. 20% higher than those of the tincture and the decoction (data not shown). Accordingly, further studies were carried out using the infusion and its associated ethyl acetate fraction.

### Antioxidant activity

The infusion of *E. telmateia* showed (Table 1) scavenging activities against DPPH and ABTS radicals (TEAC

**Table 1. Antioxidant activities and polyphenolic content of an infusion of *Equisetum telmateia* Ehrh. and the ethyl acetate fraction derived there from**

| | DPPH[a] ($EC_{50}$) | TEAC[b] | TBARS[a] ($IP_{50}$) | Total phenols[c] | Total flavonols[d] | Total flavanols[e] |
|---|---|---|---|---|---|---|
| Infusion[f,g] | 0.455 | 11.0 | 0.186 | 7.88 ± 0.70 | 0.463 ± 0.000 | 0.74 ± 0.03 |
| Ethyl acetate fraction of infusion[f,g] | 3.429 | 1.7 | 1.159 | 0.37 ± 0.06 | 0.031 ± 0.000 | 0.12 ± 0.02 |
| Infusion[f,h] | 0.119 | 50.5 | 0.041 | 36.1 ± 3.2 | 2.125 ± 0.000 | 3.39 ± 0.05 |
| Ethyl acetate fraction of infusion[f,h] | 0.018 | 342.8 | 0.006 | 78.7 ± 4.8 | 6.191 ± 0.000 | 23.2 ± 3.3 |

[a] Results expressed in mg.
[b] Results expressed as Trolox equivalent antioxidant capacity/mg of dry weight.
[c] Results (mean ± SD) expressed in g gallic acid equivalent/100 g (dry weight) of sample.
[d] Results (mean ± SD) expressed in g rutin equivalent/100 g (dry weight) of sample.
[e] Results (mean ± SD) expressed in g catechin equivalent/100 g (dry weight) of sample.
[f] All assays were performed with at least three replicates.
[g] Results expressed in terms of the dry weight of plant material extracted.
[h] Results expressed in terms of the dry weight of the infusion/ethyl acetate fraction assayed.

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assay), and protection against lipid peroxidation (TBARS assay). Such activities appeared significantly lower for the ethyl acetate fraction obtained from the infusion when the results were expressed in relation to the original dry weight of the plant sample extracted. However, this lower activity may have resulted from the presence of low concentrations of the active compounds in the plant, rather than from a low activity of the compounds present in the fraction. Thus, when the results were expressed with respect to the dry weight of the infusion and of the ethyl acetate fraction, it could be demonstrated that the ethyl acetate fraction was richer in phenolic compounds and much more active than the infusion (Table 1). The activity obtained in the DPPH assay for the ethyl acetate fraction was quite similar to that found for ascorbic acid (0.017 mg), a powerful antioxidant in biological systems, and much higher than that of a 1 mM solution of the water-soluble analogue of vitamin E, Trolox C. This confirms that the ethyl acetate fraction of *E. telmateia* possesses powerful antioxidant activity that constitutes an important contribution to the activity of the infusion.

Several reports indicate that antioxidants are beneficial to human health. The antioxidant activity detected in the ethyl acetate fraction of *E. telmateia* using *in vitro* assays supports the hypothesis that this activity can contribute to the pharmacological properties ascribed to this plant in traditional medicine. However, the correlation between *in vitro* and *in vivo* activities is not always direct since *in vivo* effects depend on the structure and physico-chemical properties of the naturally occurring compounds, which influence not only activity but also absorption, bioavailability and metabolism. In the present study, phytochemical characterisation of the polyphenol fraction in extracts of *E. telmateia* that may contribute to their biological properties has also been performed.

## Phytochemical characterisation of *E. telmateia*

The identification of polyphenols was performed using retention characteristics in RP-HPLC, through analysis of UV–vis and MS spectra, and by HPLC-PAD-ESI/MS analyses. Flavan-3-ols (i.e. catechins and proanthocyanidins) were also analysed by HPLC using detection following post-column derivatisation with DMACA.

Figure 1 shows the chromatographic profile of the ethyl acetate fraction from the infusion of *E. telmateia* recorded at 280 nm. The UV spectra suggested that the major flavonoids present in this fraction were kaempferol derivatives and flavan-3-ols. Retention characteristics and UV spectra also allowed the identification of protocatechuic and *p*-hydroxybenzoic acids, confirmed by comparison with standards, as well as the presence of various caffeic acid derivatives. Further identification of compounds was made from their MS. Table 2 summarises the retention and spectral characteristics of components detected in the HPLC-PAD-MS/MS analysis and the tentative identities assigned to these compounds.

## Flavanols

The identity of procyanidin B2 [i.e. epicatechin-(4$\beta$→8)-epicatechin; peak 4] could be confirmed by comparison with a standard previously obtained and characterised in our laboratory; the MS (molecular ion at *m/z* 579) and fragmentation pattern further substantiated this identity. The principal fragment resulting from the *retro* Diels-Alder (RDA) fission of the heterocyclic ring, characteristic of procyanidins, was observed at *m/z* 427. A fragment at *m/z* 409, corresponding to the loss of a water molecule from the *m/z* 427 fragment, and a further fragment at *m/z* 291, resulting from the cleavage of the inter-flavan linkage through the quinone-methide mechanism, were also observed. Similarly, peak 8 was identified as procyanidin trimer C1 [i.e. epicatechin-(4$\beta$→8)-epicatechin-(4$\beta$→8)-epicatechin].

In order to confirm the presence of these procyanidins in the extract, a post-column derivatisation with DMACA was performed; this reagent is specific for flavan-3-ols and produces a coloured adduct showing maximum absorption at 640 nm. Also, the ratio $A_{640}/A_{280}$ of the absorbance obtained for the peaks at 280 nm prior to the reaction and at 640 nm after the reaction can be used as a criterion for the identification of flavan-3-ols when associated with the order of elution of the compounds (Treutter *et al*., 1994). By means of these characteristics, the identities of the procyanidins B2 and

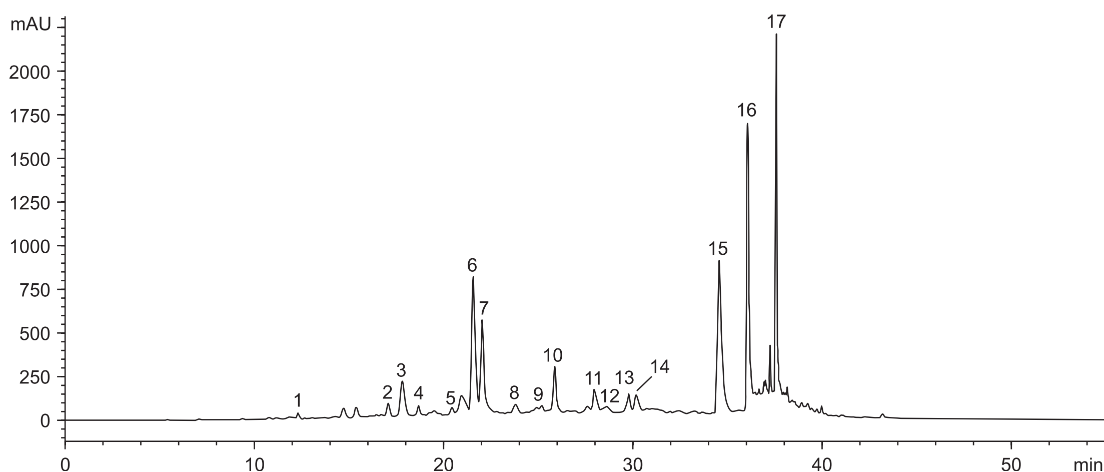


**Figure 1.** HPLC profile (detected at 280 nm) of the ethyl acetate fraction from the infusion of *Equisetum telmateia* Ehrh. (For key to peak identities see Table 2; for chromatographic protocols see the Experimental section).

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**Table 2. UV–vis and MS data and tentative identities of the compounds detected in the analysis of the ethyl acetate fraction from the infusion of *Equisetum telmateia* Ehrh**

| | | | | HPLC-ESI/MS [*m/z* (%)] | |
|---|---|---|---|---|---|
| Peak[a] | Compounds[b] | HPLC retention time (min) | $\lambda_{max}$ by HPLC-PAD (nm) | Molecular ion $[M + H]^+$ | $[M + H]^+$ fragments |
| 1 | Protocatechuic acid | 12.24 | 260, 292 | — | — |
| 2 | *p*-Hydroxybenzoic acid | 16.97 | 256 | — | — |
| 3 | Proanthocyanidin tetramer containing an Afz residue | 17.70 | 280 | 1139 | — |
| 4 | Procyanidin dimer B2 | 18.57 | 280 | 579 | 291 (13), 409 (85), 427 (100) |
| 5 | Caffeic acid derivative | 20.35 | 292sh, 324 | — | — |
| 6 | A-type proanthocyanidin trimer | 21.47 | 280 | 865 | 533 (87), 695 (72), 713 (100) |
| 7 | Caffeic acid derivative | 21.96 | 302sh, 328 | — | — |
| 8 | Procyanidin trimer C1 | 23.75 | 280 | 867 | 287 (63), 409 (58), 577 (100), 715 (46) |
| 9 | Proanthocyanidin trimer (epi)Afz-(epi)C-(epi)C | 25.15 | 280 | 851 | 561 (100), 579 (79), 715 (51), 407 (50) |
| 10 | Kaempferol acetyl-dihexose | 25.82 | 266, 286sh, 348 | 653 | 287 (93), 449 (100), 653 (12) |
| 11 | Proanthocyanidin trimer (epi)C-(epi)C-(epi)Afz | 27.95 | 282, 312 | 851 | 258 (71), 327 (69), 561 (100), 578 (78) |
| 12 | Proanthocyanidin tetramer containing two Afz residues | 28.61 | 280 | 1123 | — |
| 13 | Kaempferol glucoside-rhamnoside | 29.77 | 266, 282sh, 322sh, 348 | 595 | 445 (100) |
| 14 | Caffeic acid derivative | 30.18 | 298sh, 328 | | — |
| 15 | Kaempferol acetylglucoside-rhamnoside | 34.57 | 266, 292sh, 348 | 637 | 491 (100), 433 (67), 287 (41) |
| 16 | Kaempferol 3-*O*-glucoside | 36.07 | 266, 294sh, 342 | 449 | 287 (24), 329 (63), 385 (64), 431 (100) |
| 17 | Kaempferol 3-*O*-acetylglucoside | 37.58 | 266, 294sh, 342 | 491 | 287 (100), 329 (7), 473 (5) |

[a] Peak numbering as shown in Fig. 1.
[b] Afz, afzelechin; B2, EC-(4→8)-EC; C1, EC-(4→8)-EC-(4→8)-EC; C, catechin; EC, epicatechin, (epi)C, epicatechin or catechin; (epi)AFZ, epiafzelechin or afzelechin.

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C1 were confirmed through retention times and also from their $A_{640}/A_{280}$ ratios (1.65 and 1.67, respectively). An estimation of the amounts of flavan-3-ols by the DMACA method indicated that proanthocyanidins B2 and C1 corresponded to about 15% of the total proanthocyanidins extracted.

Peak 6 showed a UV spectrum similar to peak 8, but its molecular ion (*m/z* 865) and the main fragment from the RDA fission (*m/z* 713) gave values that were two units less. This strongly suggested that the molecule could be a proanthocyanidin trimer showing an additional ether linkage (A-type linkage) between two of its elementary (epi)catechin units. The fact that it eluted before the trimer C1 in spite of the A-type linkage (that would be expected to increase the retention time) suggested that one or several of its subunits were catechin (decreased retention time compared with epicatechin).

MS analysis of peak 9 gave a molecular ion at *m/z* 851, i.e. 16 units lower than that expected for a procyanidin trimer (Fig. 2), suggesting that it could be made up of two (epi)catechin units and one (epi)afzelechin unit. In the $MS^2$, a fragment was found at *m/z* 579 that corresponded to the loss of the (epi)afzelechin unit (272 mass units). The fragment at *m/z* 715 could be explained by an RDA fission $M^+$-136 from the (epi)afzelechin, whilst the fragment at *m/z* 561 probably resulted from the loss of a water molecule from the *m/z* 579 fragment. RDA fission usually occurs in the upper proanthocyanidin subunit (de Pascual-Teresa *et al*., 2000; Friedrich *et al*., 2000), thus indicating that (epi)afzelechin might be at that position. The same molecular ion at *m/z* 851 was obtained for peak 11, suggesting the presence of similar elementary units as for peak 9; although no RDA fragment was found, the fact that an (epi)catechin dimer (*m/z* at 578) was released indicated that (epi)afzelechin was present as the lower subunit.

The MS data of compounds associated with peaks 3 and 12 also suggested that they were heterogeneous proanthocyanidins (tetramers) composed of three (epi)catechin units linked to one (epi)afzelechin unit, and of two (epi)catechin units linked to two (epi)afzelechin units, respectively. However, the lack of $MS^2$ information prevents further speculation concerning their identities.

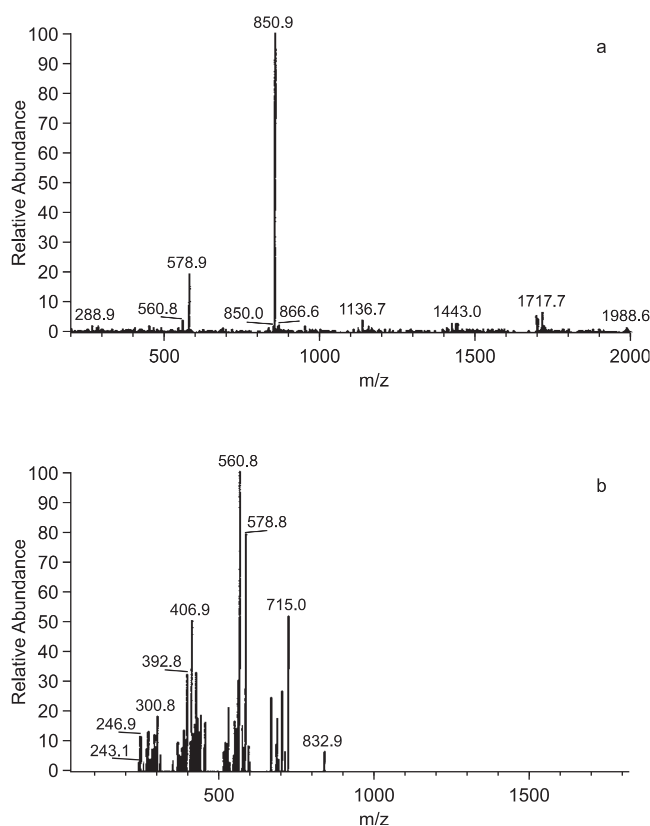


**Figure 2.** Mass spectrum (a) and $MS^2$ spectrum of the molecular ion (b) of the compound associated with peak 9 shown in Fig. 1.

A significant feature of the ethyl acetate fraction from the infusion of *E. telmateia* was the unusual absence of flavan-3-ol monomers as indicated by the lack of peaks with short retention times in the chromatographic profile obtained after reaction with DMACA (Fig. 3), and further confirmed from the HPLC-MS chromatogram when screened for molecular ions of afzelechin, catechin and gallocatechin (*m/z* at 275, 291 and 307, respectively). The absence of flavan-3-ol monomers (again confirmed by HPLC-DMACA analysis) was also a compositional feature of the infusion itself.

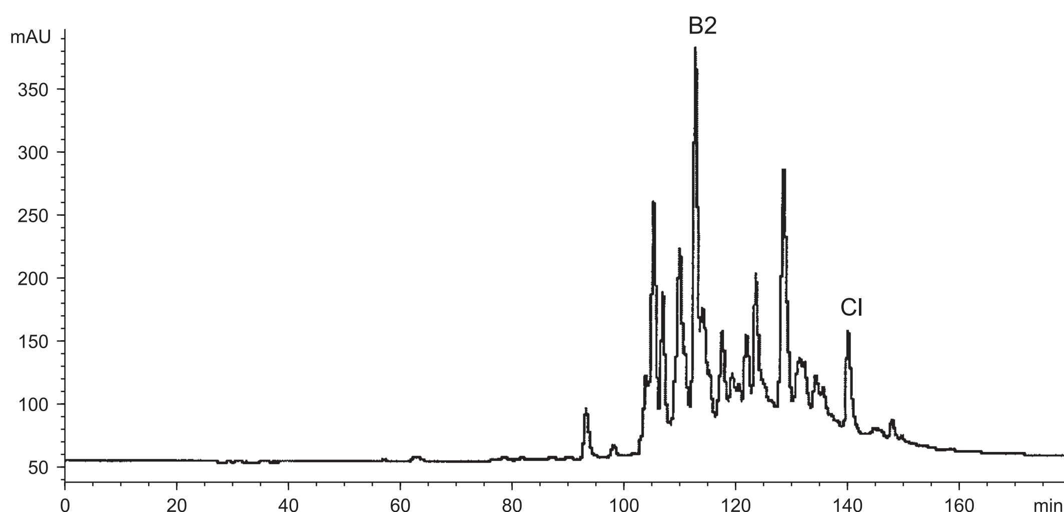


**Figure 3.** HPLC chromatogram [detection at 640 nm after reaction with *p*-dimethylaminocinnamaldehyde (DMACA)] of the ethyl acetate fraction of the infusion of *Equisetum telmateia* Ehrh. (For key to peak identities see Table 1; for chromatographic protocols see the Experimental section).

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In conclusion, the flavan-3-ol composition of the ethyl acetate fraction was characterised by proanthocyanidin oligomers, among which the presence of derivatives containing (epi)afzelechin sub-units (i.e. propelargonidins) and A-type linkages was a noteworthy characteristic. The presence of proanthocyanidins in *E. telmateia* is reported here for the first time. Furthermore, the detection of propelargonidins in this plant is particularly important as they probably account for part of the recognised anti-inflammatory action of this plant. Although much research emphasis has been given to the B-type procyanidins, some authors attribute significant anti-inflammatory and anti-bacterial activities to propelargonidins and to A-type procyanidins, respectively (Min *et al.*, 1999; Tomás-Barberán *et al.*, 2001). However, further studies are required in order to validate this hypothesis. These compounds are not frequently encountered in nature and additional studies are essential to verify their actions.

## Flavonols

Flavonols were also present in the ethyl acetate fraction from the infusion of *E. telmateia.* Peaks 10, 13, 15, 16 and 17 were identified as kaempferol derivatives according to their UV spectra and the presence in their $MS^2$ of a fragment at *m/z* 287 corresponding to kaempferol aglycone. Analysis of the MS associated with peak 13 showed an *m/z* value at 595. Clearly, a collision energy of 45 V was not enough to fragment this compound (Table 2), and therefore a collision energy of 55 V was employed. The resulting MS profiles (Fig. 4) showed fragments at *m/z* 433 and 449 corresponding to the loss of glucose and rhamnose residues, respectively. The presence of these two ions, the high intensity of the fragment at *m/z* 433 and the UV spectrum, suggested that each sugar moiety was not attached one to another as a disaccharide but rather linked at different positions in the aglycone structure. Furthermore, comparison with a standard ruled out the possibility of kaempferol 3-*O*-rutinoside. Thus, the compound was tentatively assigned to be kaempferol glucoside–rhamnoside, although no information about the substitution positions of the sugars could be obtained.

Compound 15 gave a molecular ion at *m/z* 637 (Fig. 5), i.e. 42 mass units (matching an acetyl moiety) higher than the compound associated with peak 13. An increase in the retention time of peak 15 in relation to peak 13 was also consistent with acetylation in the former compound (Veit *et al.*, 1995). The observation of a fragment at *m/z* 433 in the $MS^2$ further suggested that the acetyl residue was attached to the glucose moiety. For these reasons the compound was tentatively identified as kaempferol acetylglucoside–rhamnoside.

Compound 16 showed a molecular ion at *m/z* 449 consistent with a kaempferol glucoside, whilst its UV spectrum ($\lambda_{max}$ at 266 and 342 nm) and chromatographic elution behaviour allowed its identification as kaempferol 3-*O*-glucoside. In the same way peak 17, with a *m/z* value at 491 (42 m.u. higher than the compound associated with peak 16) and higher retention time, could be assigned to kaempferol 3-*O*-acetylglucoside.

Analysis of the MS of the compound associated with peak 10 showed a molecular ion at *m/z* 653 and fragments at *m/z* 449 and 287, corresponding to the loss

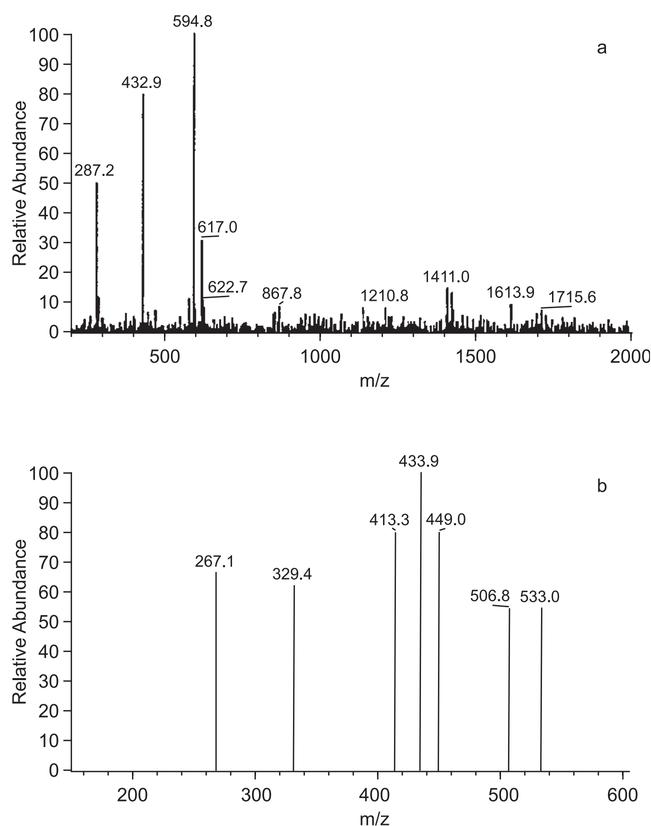


**Figure 4.** Mass spectrum (a) and $MS^2$ spectrum of the molecular ion (b) of the compound associated with peak 13 shown in Fig. 1 (collision energy 55 V).

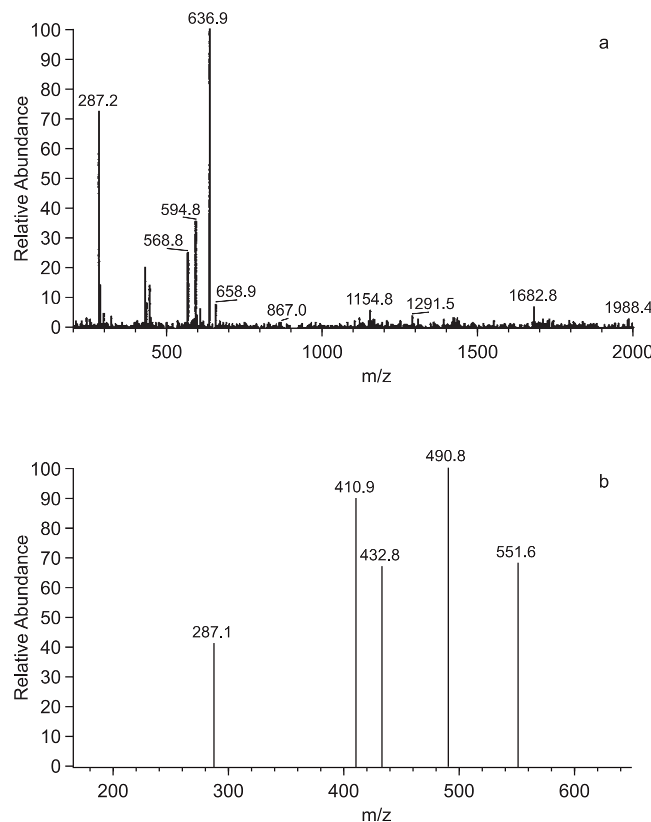


**Figure 5.** Mass spectrum (a) and $MS^2$ spectrum of the molecular ion (b) of the compound associated with peak 15 shown in Fig. 1.

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of acetyl-hexose and acetyl-dihexose residues, respectively. These results suggested that the compound was a kaempferol acetyl-dihexose derivative, an identity that was consistent with its chromatographic behaviour since, as a general rule, a higher degree of glycosylation leads to a shorter retention time (Llorach *et al*., 2003).

Although the identifications of compounds 10, 13, 15 and 16 is still tentative and further research is needed to characterise the sugar moieties, the acetyl linkages and the positions of attachment, the results from the MS and elution sequence of these compounds are in agreement with those for kaempferol derivatives, as reported by Veit *et al*. (1995).

It is important to note the high levels of kaempferol acetyl glycoside derivatives in the ethyl acetate from the infusion of *E. telmateia.* Some studies have reported anti-inflammatory activity for flavonoids, and kaempferol has been found to be a potent inhibitor of iNOS induction and a cNOS stimulator producing anti-inflammatory and anti-atherogenic activities, respectively (Olszanecki *et al*., 2002). Additionally, the well-documented antioxidant activity of this flavonol could also contribute to its anti-atherogenic activity. Recently, Backhouse *et al*. (2002) ascribed significant anti-pyretic and anti-inflammatory activities to an extract containing an acetylglucosyl derivative of kaempferol and (–)-epicatechin. Therefore, it is possible that the traditional anti-inflammatory uses of *E. telmateia* are due, at least in part, to the high content of such kaempferol derivatives.

## Acknowledgements

The authors wish to express their thanks to the Fundação para a Ciência e Tecnologia (FCT) and to the Programa Operacional de Ciência, Tecnologia e Inovação for financial support. The authors are also grateful to Segredo da Planta, Portugal, for the provision of plant material, to Dr. J. Paiva for classification of plant material, and to Dr. Joaquín Pérez-Alonso and Susana González-Manzano for technical assistance.

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