



## Chemical, biochemical and electrochemical assays to evaluate phytochemicals and antioxidant activity of wild plants

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### ABSTRACT

Plants are a source of compounds that may be used as pharmacologically active products. *Cytisus multiflorus*, *Filipendula ulmaria* and *Sambucus nigra* have been used as important medicinal plants in the Iberian Peninsula for many years, and are claimed to have various health benefits. Herein, the phytochemical composition and antioxidant activity of the mentioned wild medicinal plants were evaluated *in vitro*, based on chemical, biochemical and electrochemical methods. *F. ulmaria* was found to be richest in antioxidant phytochemicals, such as phenolics (228 mg GAE/g DW), flavonoids (62 mg CE/g DW), ascorbic acid (2700 µg/g DW) and tocopherols (497 µg/g DW). The antioxidant activity was found to vary in the order: *F. ulmaria* > *S. nigra* > *C. multiflorus*, irrespective of the analysis method. Electrochemical methods have proven to be rapid and inexpensive techniques to characterise the antioxidant activity of plant extracts.

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### 1. Introduction

Reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide, hydroxyl, acyl and alkoxyl radicals are highly reactive species that are responsible for many cell disorders through their action on proteins, lipids and DNA. Along with other pro-oxidants, such as Cu and Fe ions, ROS act by modifying the oxidative balance within cells and thus are important mediators of cell injuries. They are assumed to play an important role in the development of many diseases, such as atherosclerosis, reperfusion injury, cataractogenesis, rheumatoid arthritis, inflammatory disorders, cancer and the ageing process itself (Gutteridge & Halliwell, 2000; Halliwell, 1996; Valko et al., 2007). Natural antioxidants protect the human body from free radicals, prevent oxidative stress and associated diseases (Ferreira, Barros, & Abreu, 2009; López et al., 2007). For these reasons they play a very important role in health care. Plants are a source of compounds with antioxidant activity such as phenolic acids, flavonoids (including anthocyanins and tannins), vitamins and carotenoids that may be used as pharmacologically active products (López et al., 2007).

Widespread empirical use of wild plants demands accurate and reliable information on their phytochemicals and antioxidant activity, as well as on the potential benefits and prospective products, such as nutraceuticals and phytomedicines. This is the case of three species that have long been used in the Iberian Peninsula as

important medicinal plants and less often as food or food additives. *Cytisus multiflorus* (L'Hér.) Sweet (Fabaceae, white Spanish broom; port: giesta branca) which is native to the Iberian Peninsula; *Filipendula ulmaria* (L.) Maxim. (Rosaceae, meadowsweet; port: rainha-dos-prados) and *Sambucus nigra* L. (Adoxaceae, elder; port: sabugueiro), are native throughout most of Europe and Asia. Besides the symbolic and aesthetic value of their whitish flowers, they have been regarded as powerful ingredients for homemade remedies mainly due to their anti-inflammatory, diuretic and diaphoretic properties, traditionally recognised by healers and consumers. A sweet tea is made from the dried flowers of meadowsweet; elderflowers are eaten raw or cooked and are used to prepare syrups (Camejo-Rodrigues, Ascensão, Bonet, & Vallès, 2003; Carvalho, 2010; Pardo de Santayana, 2007, 2008).

These plants represent a source of natural antioxidants that might serve as leads for the development of novel drugs. In fact, several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to act through an antioxidant and/or radical-scavenging mechanism as part of their activity (Conforti et al., 2008). Furthermore, some drugs have various and severe adverse effects. Therefore, products of natural origin with no or very few side effects are desirable as substitutes for chemical therapeutics.

Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively (Frankel & Finley, 2008; Prior, Wu, & Schaich, 2005). With respect to electrochemical

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methods, there has been considerable work done on characterising the behaviour of isolated natural products. In particular, voltammetric methods represent an attractive option for rapid screening of large numbers of plant samples in the search for novel antioxidants. However, the technique has not yet found widespread use, when compared to established methods, such as the DPPH and Folin–Ciocalteu assays. In fact, there are limited references on the application of voltammetric techniques on plant or algae extracts, juices, tea and wine, with the aim of assessing their total antioxidant activity (Chevion, Chevion, Chock, & Beecher 1999; Kilmartin, Zou, & Waterhouse, 2001; Litescu & Radu, 2000).

Herein the antioxidant capacity of these three wild medicinal plants was evaluated by radical-scavenging activity and lipid peroxidation inhibition, using *in vitro* assays. Electrochemical techniques, such as cyclic voltammetry and differential pulse voltammetry were also used, to provide a further insight into redox-processes within plant extracts. These techniques have been tested and developed as an alternative and/or complementary tool for the evaluation of antioxidant activity, expressed in terms of “antioxidant power”, due to their quickness, simplicity and low cost (Barros et al., 2008; Blasco, Rogerio, González, & Escarpa, 2005; Cosio, Buratti, Mannino, & Benedetti, 2006). The aim of this study was to obtain a complete characterisation of the antioxidant properties of flowers of Spanish broom, meadowsweet and elder, and their chemical composition in phytochemicals and antioxidants, such as vitamins, pigments, sugars, fatty acids, phenolics and flavonoids.

## 2. Materials and methods

### 2.1. Samples

Inflorescences of the three species, with flowers fully open and functional, were collected in May 2009, in the Natural Park of Montesinho, Trás-os-Montes, north-eastern Portugal, considering the Portuguese folk pharmacopeia and the local medicinal uses. Voucher specimens were deposited in the Herbário da Escola Superior Agrária de Bragança (BRESA). Samples were lyophilised (Ly-8-FM-ULE, Snijders Scientific B.V., Tilburg, The Netherlands) and stored at  $-20^{\circ}\text{C}$  for subsequent analysis.

### 2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95%, ethyl acetate 99.8% and methanol were of HPLC grade from Lab-Scan (Lisbon, Portugal). The fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO), as were other individual fatty acid isomers, L-ascorbic acid, tocopherols and sugars standards, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-catechin. Racemic tocol 50 mg/mL, was purchased from Matreya LLC (Pleasant Gap, PA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA). Sodium perchlorate monohydrate, purity grade, was purchased from Fluka (Buchs, Switzerland), and was dried overnight at  $30^{\circ}\text{C}$  before use. All other chemicals and solvents were of analytical grade and purchased from chemical suppliers. Ultra-pure water was obtained from a Milli-Q water purification system (TGI Pure Water Systems; IsoPure Water, Santa Ana, CA).

### 2.3. Phytochemicals and antioxidants

#### 2.3.1. Determination of tocopherols

BHT solution in hexane (10 mg/mL, 100  $\mu\text{L}$ ) and tocol solution in hexane (internal standard; 50  $\mu\text{g}$ /mL, 400  $\mu\text{L}$ ) were added to the

sample prior to the extraction procedure. The samples ( $\sim 500$  mg) were homogenised with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenised (1 min), centrifuged (Centurion K240R refrigerated centrifuge, 5 min, 4000g; Centurion Scientific Ltd., Stoughton, UK) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2- $\mu\text{m}$  nylon filters from Whatman, transferred into a dark injection vial and analysed by HPLC. The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000; Knauer, Berlin), degasser system (Smartline Manager 5000), auto-sampler (AS-2057 Jasco, Tokyo, Japan) and a fluorescence detector (FP-2020; Jasco) with 290 and 330 nm as excitation and emission wavelengths, respectively. Data were analysed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved with a Polyamide II (250  $\times$  4.6 mm) normal-phase column from YMC (Waters, Milford, MA) operating at  $30^{\circ}\text{C}$ . The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20  $\mu\text{L}$ . The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherol contents in the samples are expressed in  $\mu\text{g}$  per g of dry sample (DW).

#### 2.3.2. Determination of ascorbic acid

A fine powder (20 mesh) of sample (500 mg) was extracted with metaphosphoric acid (1%, 10 mL) for 45 min at room temperature and filtered through Whatman N4 filter paper. The filtrate (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance was measured after 30 min at 515 nm against a blank (Analytik Jena 200-2004 spectrophotometer; Analytik Jena, Jena, Germany). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid ( $6.0 \times 10^{-3}$  –  $1.0 \times 10^{-1}$  mg/mL), and the results were expressed as mg per 100 g DW.

#### 2.3.3. Determination of lipid-soluble pigments

A fine dried powder (150 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Content of  $\beta$ -carotene was calculated according to the following equation:

$$\begin{aligned} \beta\text{-carotene (mg/100 mL)} &= 0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}; \\ \text{Lycopene (mg/100 mL)} &= -0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}; \\ \text{Chlorophyll a (mg/100 mL)} &= 0.999 \times A_{663} - 0.0989 \times A_{645}; \\ \text{Chlorophyll b (mg/100 mL)} &= -0.328 \times A_{663} + 1.77 \times A_{645}, \text{ and} \\ &\text{further expressed in } \mu\text{g per g dry weight (dw)}. \end{aligned}$$

#### 2.3.4. Determination of sugars

Dried sample powder (1.0 g) was spiked with melezitose (IS, 5 mg/mL), and was extracted with 40 mL of 80% aqueous ethanol at  $80^{\circ}\text{C}$  for 30 min. The resulting suspension was centrifuged at 15,000g for 10 min. The supernatant was concentrated at  $60^{\circ}\text{C}$  under reduced pressure and defatted three times with 10 mL of ethyl ether. After concentration at  $40^{\circ}\text{C}$ , the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2- $\mu\text{m}$  nylon filters from Whatman (Maidstone, UK). Soluble sugars were determined by HPLC coupled to a refraction index

(RI) detector (Knauer Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5  $\text{NH}_2$  column ( $4.6 \times 250$  mm, 5  $\mu\text{m}$ , Knauer) operating at 30 °C. The mobile phase was acetonitrile/deionised water, 7:3 (v/v) at a flow rate of 1.0 mL/min. Sugar identification was made by comparing the relative retention times of sample peaks with standards. Quantification was made by internal normalisation of the chromatographic peak area and the results are expressed in mg per g DW.

### 2.3.5. Determination of fatty acids

Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v), for at least 12 h in a stirrer bath at 50 °C and 160 rpm; then 3 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by shaking in a vortex, and the upper phase was passed through a micro-column of anhydrous sodium sulphate, in order to eliminate the water; the sample was recovered in a Teflon vial, and before injection the sample was filtered with a 0.2- $\mu\text{m}$  nylon filter from Whatman. The fatty acid profile was analysed with a DANI Model 1000 gas chromatograph (DANI Instruments S.p.A, Cologno Monzese, Italy) equipped with a split/splitless injector, a flame ionisation detector (FID) and a Macherey–Nagel column (30 m  $\times$  0.32 mm ID  $\times$  0.25  $\mu\text{m}$  film thickness; Macherey–Nagel GmbH & Co. KG, Düren, Germany). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (40:1) was carried out at 250 °C. For each analysis 1  $\mu\text{L}$  of the sample was injected onto the GC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

### 2.3.6. Determination of phenolics

A fine dried powder (20 mesh;  $\sim 1$  g) was stirred with 50 mL of methanol at 25 °C and 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 50-mL portion of methanol. The combined methanolic extracts were evaporated at 35 °C under reduced pressure, redissolved in methanol at 20 mg/mL (stock solution), and stored at 4 °C for further use. For phenolics determination, an aliquot of the extract solution (0.125 mg/mL; 1 mL) was mixed with Folin–Ciocalteu reagent (5 mL, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve ( $9.4 \times 10^{-3}$ – $1.5 \times 10^{-1}$  mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g DW.

### 2.3.7. Determination of flavonoids

For flavonoids, an aliquot of the extract solution (0.125 mg/mL; 0.5 mL) was mixed with distilled water (2 mL) and subsequently with  $\text{NaNO}_2$  solution (5%, 0.15 mL). After 6 min,  $\text{AlCl}_3$  solution (10%, 0.15 mL) was added and allowed to stand for a further 6 min; thereafter, NaOH solution (4%, 2 mL) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve ( $4.5 \times 10^{-3}$ – $2.9 \times 10^{-1}$  mg/mL) and the results were expressed as mg of (+)-catechin equivalents (CE) per g DW.

## 2.4. Chemical and biochemical assays to evaluate antioxidant activity

### 2.4.1. DPPH radical-scavenging activity

The reaction mixture in each one of the 96 wells (ELX800 microplate reader; BioTek, Winooski, VT) consisted of one of the different concentrations of the extracts (0.03–1.00 mg/mL; 30  $\mu\text{L}$ ) and aqueous methanolic solution (80:20 v/v, 270  $\mu\text{L}$ ) containing DPPH radicals ( $6 \times 10^{-5}$  M). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation:

$$\% \text{RSA} = [(A_{\text{DPPH}} - A_s)/A_{\text{DPPH}}] \times 100,$$

where  $A_s$  is the absorbance of the solution when the sample extract has been added at a particular level, and  $A_{\text{DPPH}}$  is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity ( $EC_{50}$ ) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

### 2.4.2. Reducing power

The different concentrations of the extracts (0.03–1.00 mg/mL; 0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was transferred to 48-well plates, and deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL) were added. The absorbance was measured at 690 nm in the microplate reader mentioned above. The extract concentration providing 0.5 of absorbance ( $EC_{50}$ ) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

### 2.4.3. Inhibition of $\beta$ -carotene bleaching

A solution of  $\beta$ -carotene was prepared by dissolving  $\beta$ -carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottomed flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.03–1.00 mg/mL; 0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. A blank, devoid of  $\beta$ -carotene, was prepared for background subtraction.  $\beta$ -Carotene bleaching inhibition was calculated using the following equation:

$$\beta\text{-carotene bleaching inhibition} = (\beta\text{-carotene content after } 2 \text{ h of assay} / \text{initial } \beta\text{-carotene content}) \times 100.$$

The extract concentration providing 50% antioxidant activity ( $EC_{50}$ ) was calculated by interpolation from the graph of  $\beta$ -carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

### 2.4.4. Inhibition of lipid peroxidation using thiobarbituric acid-reactive substances (TBARS)

Brains were obtained from pig (*Sus scrofa*) of body weight  $\sim 150$  kg, dissected and homogenised in ice-cold Tris–HCl buffer (20 mM, pH 7.4) using a Polytron, to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the

different concentrations of the extracts (0.03–1.00 mg/mL; 0.2 mL) in the presence of FeSO<sub>4</sub> (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio(\%)} = [(A - B)/A] \times 100,$$

where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC<sub>50</sub>) was calculated from the graph of TBARS inhibition percentage against extract concentration. Trolox was used as standard.

## 2.5. Electrochemical assays to evaluate antioxidant activity

### 2.5.1. Sample preparation

A lyophilised fine powder of plant flowers (20 mesh; 1 g) was extracted with 50 mL methanol in the refrigerator (~4 °C) for 24 h, in the dark and without stirring. After extraction, samples were filtered in a glass funnel through glass wool. For each sample, an aliquot of 10 mL of filtered extract was transferred to a vial containing NaClO<sub>4</sub>, so that the final concentration of support electrolyte was 0.1 M. Solutions were analysed immediately.

### 2.5.2. Instrumentation

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on an Autolab PGSTAT 302 potentiostat/galvanostat (Metrohm Autolab B.V., Utrecht), using a closed standard three electrode cell. A glassy carbon electrode (MF-2012,  $\phi$  = 3.0 mm; BASi, Lafayette, IN) was used as working electrode and Pt foil as counter electrode. All potentials refer to an Ag/AgCl 3 M KCl (+207 mV vs SHE) reference electrode (Metrohm). Prior to use, the working electrode was cleaned through physical, chemical and electrochemical treatment: (i) it was polished against a Master-Tex (Bühler Ltd., London, UK) polishing pad wetted with an aqueous suspension of 0.3-µm alumina (Bühler), (ii) sonicated in 6 M HCl for 1 min, and then in methanol, and (iii) submitted to an anodic current of 2.0 V for 60 s. The electrode was thoroughly rinsed with deionised water and methanol between every step, and blotted dry. This is important because polyphenolic compounds tend to strongly adsorb at the surface of glassy carbon electrodes.

### 2.5.3. Procedure

All extracts and standard compounds were studied in methanolic 0.1 M NaClO<sub>4</sub>. All the solutions were analysed immediately after preparation and the electrochemical responses recorded after immersion of the glassy carbon electrode, to minimise adsorption of species onto the electrode surface prior to run. Cyclic voltammetry (CV) was used to characterise the electrochemical responses between –0.5 and +1.5 V, at 100 mV/s, whereas the antioxidant power was evaluated by differential pulse voltammetry (DPV), using the following operating conditions: 60 mV pulse amplitude and 20 mV/s scan rate.

### 2.5.4. Quantification

A calibration curve was prepared by plotting the concentration of ascorbic acid solutions between 0.05 and 10.00 mg/mL against the current intensity of the respective DPV signals measured at

peak maxima. This curve was found to be linear in the range 0.05–1.32 mg/mL. The analytical signal of samples (current density) was measured between peak maxima and the baseline, defined as the tangent between the lowest and the highest potential valley. This current density was converted to equivalents of ascorbic acid (mg/mL), and expressed in terms of equivalents of ascorbic acid (AA) per gram of plant material (DW), assuming the total volume used in the extraction procedure (50 mL) and the sample weight. The sum of the values calculated at peak maxima for each electrochemical process was used to express the total electrochemical antioxidant power (TEAP) of the plant material.

## 2.6. Statistical analysis

The results are expressed as mean values ± standard deviation (SD). The results were analysed using one-way analysis of variance, followed by Tukey's HSD Test with  $\alpha$  = 0.05. This treatment was carried out using SPSS Version16.0 (Chicago, IL).

## 3. Results and discussion

### 3.1. Phytochemicals and antioxidants

The content in vitamins (tocopherols and ascorbic acid) and pigments (carotenoids and chlorophylls) of the three studied medicinal plants are given in Table 1. Ascorbic acid was the most abundant vitamin (>1730 µg/g DW) and  $\alpha$ -tocopherol was the major tocopherol in all the samples (>313 µg/g); *F. ulmaria* was the richest sample in  $\alpha$ -tocopherol; its tocopherols profile is shown in Fig. 1.  $\delta$ -Tocopherol was not detected in *S. nigra* and was a minor compound in the other two plants. Flowers of *F. ulmaria* and *C. multiflorus* presented the highest content of ascorbic acid (2700 µg/g and 2670 µg/g, respectively) and tocopherols (497 and 483 µg/g, respectively), without significant differences,  $p$  < 0.05, between them. Kaack and Austed (1998) reported the quantification of ascorbic acid in fresh fruits of *S. nigra* (60–250 µg/g), but nothing is reported about the concentration of ascorbic acid or tocopherols in the flowers of the three studied plants.

Vitamin E (tocopherols) and vitamin C (ascorbic acid) are naturally-occurring antioxidant nutrients that play important roles in health by inactivating harmful free radicals produced through normal cellular activity and from various stressors (Chew, 1995). Cooperative interactions exist among vitamin C and vitamin E; they interact synergistically at the membrane–cytosol interface to regenerate membrane-bound oxidised vitamin E. The interac-

**Table 1**

Composition of medicinal plants in vitamins (µg/g DW), pigments (µg/g DW) and sugars (mg/g DW) (mean ± SD;  $n$  = 9). In each row different letters mean significant differences ( $p$  < 0.05).

Compound	<i>Cytisus multiflorus</i>	<i>Filipendula ulmaria</i>	<i>Sambucus nigra</i>
$\alpha$ -tocopherol	348 ± 17.5b	425 ± 15.5a	313 ± 4.08c
$\beta$ -tocopherol	34.8 ± 4.14a	15.9 ± 0.13b	8.12 ± 0.71c
$\gamma$ -tocopherol	92.6 ± 4.51a	44.3 ± 1.66b	2.89 ± 0.18c
$\delta$ -tocopherol	7.63 ± 0.46b	11.3 ± 0.92a	nd
Total tocopherols	483 ± 9.32a	497 ± 16.1a	324 ± 4.61b
Ascorbic acid	2670 ± 12.6a	2700 ± 27.0a	1730 ± 4.79b
$\beta$ -carotene	53.9 ± 0.06a	36.3 ± 0.17b	18.4 ± 0.05c
Lycopene	11.3 ± 0.04a	6.18 ± 0.08b	5.34 ± 0.04c
Chlorophyll a	60.3 ± 0.05b	78.2 ± 0.07a	45.0 ± 0.02c
Chlorophyll b	51.6 ± 0.10a	46.7 ± 0.20b	32.0 ± 0.06c
Glucose	42.7 ± 1.40a	13.1 ± 0.61c	32.3 ± 0.66b
Fructose	69.3 ± 2.71a	19.8 ± 0.65c	26.6 ± 0.96b
Sucrose	18.3 ± 0.73b	11.3 ± 0.44c	24.7 ± 0.26a
Trehalose	7.62 ± 0.98b	20.9 ± 1.17a	nd
Total sugars	138 ± 5.82a	65.1 ± 0.53c	83.6 ± 0.92b

nd – not detected.

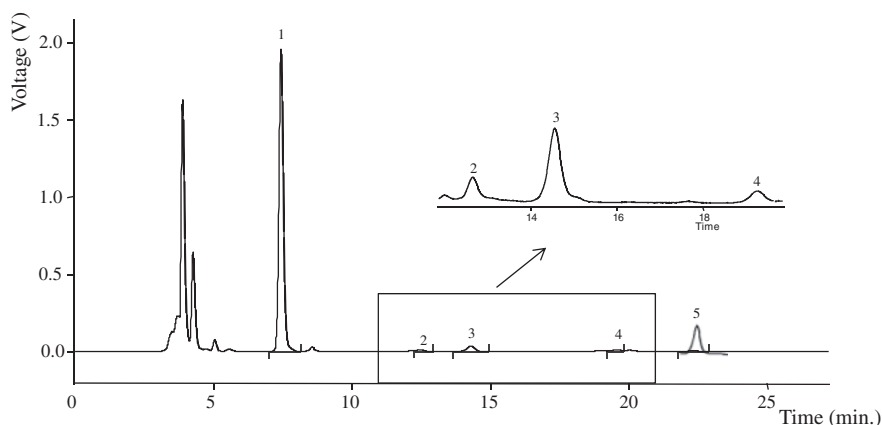


Fig. 1. Tocopherols profile of *F. ulmaria*. 1:  $\alpha$ -Tocopherol; 2:  $\beta$ -tocopherol; 3:  $\gamma$ -tocopherol; 4:  $\delta$ -tocopherol; 5: tocol (IS).

tions among these antioxidants are very important in protecting cells because the concentration of each antioxidant alone may not be adequate to effectively protect cells from lipid peroxidation (Chew, 1995; Nagaoka, Kakiuchi, Ohara, & Mukai, 2007). The presence of both vitamins could explain the empirical uses of the studied plants in oxidative stress-related diseases as previously discussed.

Carotenoids and chlorophylls were found in all the studied medicinal plants, Table 1. The concentrations of  $\beta$ -carotene (53.9  $\mu\text{g/g}$  DW), lycopene (11.3  $\mu\text{g/g}$ ) and chlorophyll b (51.6  $\mu\text{g/g}$ ) were higher in *C. multiflorus* flowers. *F. ulmaria* contained the highest levels of chlorophyll a (78.2  $\mu\text{g/g}$ ). Carotenoids are amongst nature's most widespread pigments and have also received substantial attention because of both their provitamin and antioxidant roles. The peroxy radicals ( $\text{ROO}^\bullet$ ) formed from lipids (especially polyunsaturated phospholipids) are very damaging to cells. The extensive systems of double bonds make carotenoids susceptible to attack by peroxy radicals, resulting in the formation of inactive products (Rao & Rao, 2007). Chlorophyll and its derivatives are also known to have antioxidant activity, being associated with reduced risks of diseases induced by free radicals, such as certain types of cancers. The function of chlorophyll in animals is suggested to be inhibition of lipid peroxidation and protection of mitochondria from oxidative damage induced by various free-radicals (Lanfer-Marquez, Barros, & Sinnecker, 2005). Therefore, these pigments could be very important antioxidants.

The three wild medicinal plants contained glucose, fructose, sucrose and trehalose as main sugars (Table 1). Fructose predominates in *C. multiflorus*, trehalose was the most abundant sugar in

*F. ulmaria* and glucose predominates in *S. nigra*. *C. multiflorus* flowers revealed the highest total sugars content (138 mg/g DW), with the highest levels of glucose (42.7 mg/g) and fructose (69.3 mg/g), both of them reducing sugars. The sugars profile of this sample is shown in Fig. 2. Veberic, Jakopic, Stampar, and Schmitzer (2009) reported the concentrations of individual sugars in fruits of *S. nigra* (68.5–104 mg/g FW) with fructose, glucose and sucrose as main sugars. Other authors described the levels of glucose (2.02 g/L) and sucrose (1.79 g/L) in concentrated juices of *S. nigra* fruits (Sadilova, Stintzing, Kammerer, & Carle, 2009). Nonetheless, nothing is reported about their concentrations in any of the flowers studied herein.

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of the three wild medicinal plants are shown in Table 2. Twenty-four fatty acids were identified and quantified. The major fatty acids found were  $\alpha$ -linolenic acid (C18:3 *n*-3; 25–33%) followed by linoleic acid (C18:2 *n*-6; 18–25%) contributing to the prevalence of PUFA in all the samples. Some authors showed that linoleic acid, conjugated linoleic acid, and linoleic methyl ester have antioxidative activity and proposed this as a possible explanation for anticarcinogenic and antiatherogenic effects. Nevertheless, additional studies are necessary to show their free radical-scavenging activity in different radical systems and under physiological conditions, and to determine whether there is any link between their radical-scavenging properties and their biological effects (Fagali & Catalá, 2008).

Palmitic acid was the most abundant SFA in all samples (11–18%). This fatty acid was the major compound found in pollen of

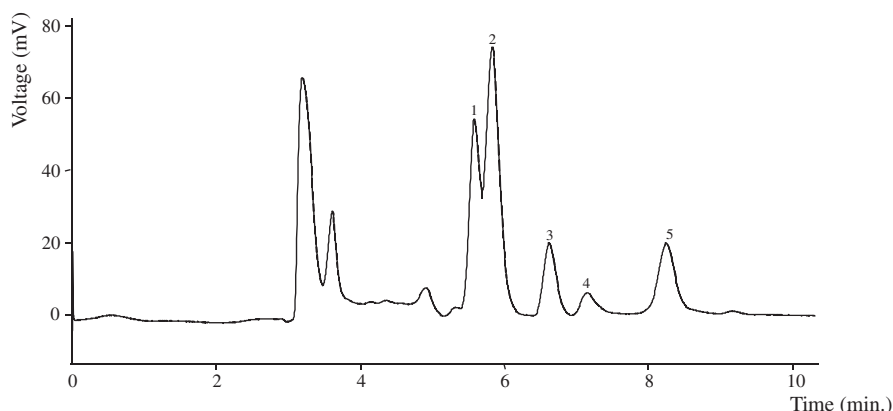


Fig. 2. Sugars profile of *C. multiflorus*. 1: Fructose; 2: glucose; 3: sucrose; 4: trehalose; 5: melezitose (IS).



*S. nigra* (35.69%), followed by C18:1 *n*-9 (14.26%), C18:2 *n*-6 (12.79%) and C18:3 *n*-3 (12.48%) (Stránský, Valterová, & Fiedler, 2001). Despite the similarities in the most abundant fatty acids in the pollen studied by those authors and in the flowers used in the present work, there are some differences in the levels found for each compound. The fatty acids profile of *S. nigra* flowers is shown in Fig. 3. As far as we know, this is the first report about the fatty acid composition in flowers of *C. multiflorus* and *F. ulmaria*.

Phenolic compounds are also important antioxidants found in the studied plants (45.6–92.7 mg GAE/g DW; Table 3); *F. ulmaria* flowers revealed the highest content in phenolics and flavonoids. This sample gave much higher total phenolic content than methanolic extracts of leaves of meadowsweet from Greece (7.2 mg/g DW; Proestos, Boziaris, Kapsokelafou, & Komaitis, 2008) and aerial parts from Finland (26.8 mg/g DW) (Kahkonen et al., 1999), and of aqueous extracts of aerial parts (flowers, stems and leaves) from Ireland submitted to different drying treatments (110–119 mg/g; Harbourne, Marete, Jacquier, and O'Riordan, 2009), temperatures (39–63 mg/g) and pH (43–57 mg/g; Harbourne, Jacquier, and O'Riordan, 2009). Flavonoids have been extracted from meadowsweet leaves using hot aqueous ethanol (70%) or methanol in a Soxhlet apparatus, and from meadowsweet flowers using hydro-alcoholic solutions and these extracts were found to contain up to 6% total flavonoids. Leaves and flowers of *F. ulmaria* are known to contain phenolic acids (glycoside derivatives of salicylaldehyde and methyl salicylate), flavonols (glycosides of kaempferol and quercetin) and ellagitannins (rugosins and tellimagradins) (Calliste, Trouillas, Allais, Simon, & Duroux, 2001; Fecka, 2009; Harbourne, Jacquier, & O'Riordan, 2009; Harbourne, Marete, Jacquier, & O'Riordan, 2009; Scheer & Wichtl, 1987). Infusions of its flowers and strained decoctions of its underground rhizomes are highly recommended to treat pneumonia, flu, urinary tract infections, rheumatism and headache (Carvalho, 2010; Pardo de Santayana, 2008). In fact, meadowsweet has been traditionally used to treat various ailments due to its antipyretic, diuretic, analgesic and anti-inflammatory properties.

Elderberry, the berry of *S. nigra* (19.5 mg GAE/g FW; Wu, Gu, Prior, & McKay, 2004) and particularly elderberry wine has been found to contain higher concentrations of phenolics than red wine (1753 mg GAE/L) (Rupasinghe & Clegg, 2007). The anticarcinogenic and antioxidative effect of elderberry juice has also been attributed to the high content of anthocyanins and other flavonoids (Kaack & Austed, 1998). A low-calorie juice cocktail (including elderberry) rich in anthocyanins was successfully used in a weight loss program in obese men (Chrubasik et al., 2008). Furthermore, the con-

**Table 2**

Composition (%) of the medicinal plants in fatty acids (mean  $\pm$  SD; *n* = 9). In each column different letters mean significant differences (*p* < 0.05).

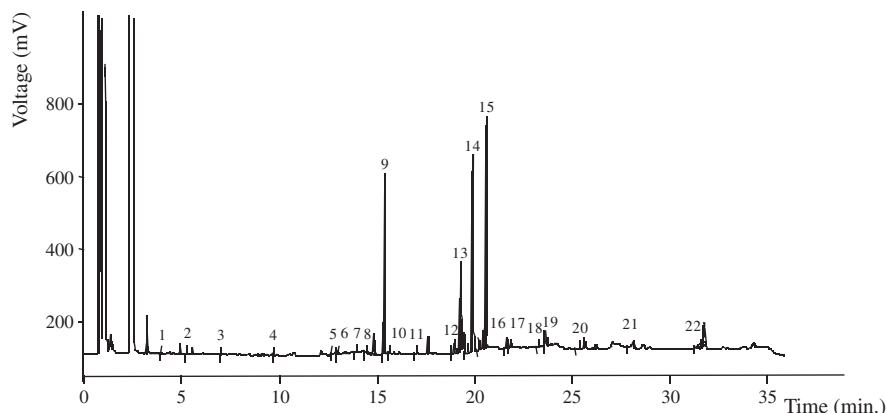
Fatty acid	<i>Cytisus multiflorus</i>	<i>Filipendula ulmaria</i>	<i>Sambucus nigra</i>
C6:0	0.10 $\pm$ 0.01	2.16 $\pm$ 0.11	0.03 $\pm$ 0.00
C8:0	0.06 $\pm$ 0.00	0.19 $\pm$ 0.01	0.02 $\pm$ 0.00
C10:0	0.04 $\pm$ 0.00	0.06 $\pm$ 0.00	0.02 $\pm$ 0.00
C12:0	0.33 $\pm$ 0.00	0.73 $\pm$ 0.01	0.14 $\pm$ 0.00
C14:0	0.53 $\pm$ 0.02	10.05 $\pm$ 0.53	0.42 $\pm$ 0.03
C14:1	0.05 $\pm$ 0.00	0.11 $\pm$ 0.01	0.05 $\pm$ 0.00
C15:0	0.13 $\pm$ 0.01	0.12 $\pm$ 0.00	0.15 $\pm$ 0.00
C15:1	nd	nd	2.07 $\pm$ 0.41
C16:0	11.14 $\pm$ 0.02	12.22 $\pm$ 0.03	18.06 $\pm$ 0.53
C16:1	0.60 $\pm$ 0.02	nd	0.36 $\pm$ 0.04
C17:0	0.37 $\pm$ 0.02	0.37 $\pm$ 0.03	0.28 $\pm$ 0.02
C18:0	4.09 $\pm$ 0.02	3.93 $\pm$ 0.13	2.02 $\pm$ 0.17
C18:1 <i>n</i> -9 <i>cis</i>	6.59 $\pm$ 0.76	2.28 $\pm$ 0.10	11.93 $\pm$ 0.16
C18:2 <i>n</i> -6 <i>cis</i>	24.31 $\pm$ 0.28	18.02 $\pm$ 0.58	24.64 $\pm$ 0.49
C18:3 <i>n</i> -6	nd	0.11 $\pm$ 0.01	nd
C18:3 <i>n</i> -3	32.49 $\pm$ 1.11	32.89 $\pm$ 0.47	25.43 $\pm$ 1.73
C20:0	3.21 $\pm$ 0.08	3.48 $\pm$ 0.25	1.26 $\pm$ 0.10
C20:1 <i>cis</i>	0.47 $\pm$ 0.02	0.32 $\pm$ 0.05	0.81 $\pm$ 0.02
C20:2 <i>cis</i>	0.18 $\pm$ 0.02	0.06 $\pm$ 0.00	nd
C20:3 <i>n</i> -3 + C21:0	0.36 $\pm$ 0.01	1.10 $\pm$ 0.05	0.21 $\pm$ 0.01
C20:5 <i>n</i> -3	nd	0.85 $\pm$ 0.05	0.57 $\pm$ 0.03
C22:0	1.68 $\pm$ 0.05	2.53 $\pm$ 0.09	1.99 $\pm$ 0.20
C23:0	11.26 $\pm$ 0.29	4.35 $\pm$ 0.01	1.81 $\pm$ 0.26
C24:0	2.07 $\pm$ 0.04	4.06 $\pm$ 0.14	7.74 $\pm$ 1.21
Total SFA	34.98 $\pm$ 0.33b	44.26 $\pm$ 0.67a	33.92 $\pm$ 1.47b
Total MUFA	7.71 $\pm$ 1.75b	2.71 $\pm$ 0.16c	15.23 $\pm$ 0.64a
Total PUFA	57.34 $\pm$ 1.38a	53.03 $\pm$ 0.83b	50.85 $\pm$ 2.11b

nd – not detected.

**Table 3**

Extraction yields, content in phenolics and flavonoids, and antioxidant activity ( $EC_{50}$  values, mg/mL) of the medicinal plants (mean  $\pm$  SD; *n* = 9). In each row different letters mean significant differences (*p* < 0.05).

	<i>Cytisus multiflorus</i>	<i>Filipendula ulmaria</i>	<i>Sambucus nigra</i>
Extraction yield (%)	36.3 $\pm$ 3.27a	29.3 $\pm$ 2.03b	37.7 $\pm$ 2.12a
Phenolics (mg GAE/g DW)	45.6 $\pm$ 3.29c	228 $\pm$ 0.53a	92.7 $\pm$ 4.66b
Flavonoids (mg CE/g DW)	7.86 $\pm$ 0.42c	62.4 $\pm$ 4.83a	26.2 $\pm$ 0.51b
DPPH-scavenging activity	2.02 $\pm$ 0.11a	0.05 $\pm$ 0.01c	0.57 $\pm$ 0.03b
Reducing power	0.64 $\pm$ 0.02a	0.06 $\pm$ 0.00c	0.27 $\pm$ 0.01b
$\beta$ -Carotene bleaching inhibition	0.35 $\pm$ 0.01a	0.09 $\pm$ 0.01c	0.16 $\pm$ 0.01b
TBARS inhibition	0.30 $\pm$ 0.02a	0.05 $\pm$ 0.00c	0.12 $\pm$ 0.01b



**Fig. 3.** Fatty acids profile of *S. nigra*. 1: C6:0; 2: C8:0; 3: C10:0; 4: C12:0; 5: C14:0; 6: C14:1; 7: C15:0; 8: C15:1; 9: C16:0; 10: C16:1; 11: C17:0; 12: C18:0; 13: C18:1 *n*-9; 14: C18:2 *n*-6; 15: C18:3 *n*-3; 16: C20:0; 17: C20:1; 18: C20:3 *n*-3 + C21:0; 19: C20:5 *n*-3; 20: C22:0; 21: C23:0; 22: C24:0.

sumption of elderberry juice induces a significant rise in plasma antioxidant capacity and a significant decrease in plasma malondialdehyde *in vivo* (Netzel et al., 2002, 2005).

Flowers of *S. nigra* have been found to contain hydroxycinnamic acids, mono- and di-caffeoylquinic acid derivatives, flavones and flavonol glycosides (Christensen, Kaack, & Fretté, 2008; Dawidowicz, Wianowska, Gawdzik, & Smolarz, 2003; Rieger, Müller, Guttenberger, & Bucar, 2008). Elderflower is recommended by the German Commission E for upper respiratory tract infections for its secretolytic effect (Chrubasik et al., 2008). Its pharmacological properties result, among other things, from the presence of flavonoids (Dawidowicz, Wianowska, & Baraniak, 2006). Furthermore, elderflower extracts are used as beverage and food flavourings (Veberic et al., 2009). Different European ethnobotanical surveys document that elder can satisfy several traditional needs (Carvalho, 2010; Neves, Matosa, Moutinho, Queiroz, & Gomes, 2009; Parada, Carrió, Bonet, & Vallès, 2009; Vallès, Bonet, & Agelet, 2004). The flower heads are used in infusions to treat respiratory system affections, such as bronchitis, cough, and cold infections; they are also a very good tonic for diabetes, blood cleanser and stomachic. Externally, the decoctions are used in poultices to ease pain and reduce inflammation, or as an ointment to heal chilblains, burns and wounds. Sometimes elder infusions are seen as a panacea (Carvalho, 2010).

*Cytisus* sp. pl. have been found to contain C-glycosyl flavones (6'-*O*-acetylscoparin), flavonol glycosides (rutin, quercetin, kaempferol, isorhamnetin, quercitrin) and isoflavones (genistein, sarothamnocide) (Brum-Bousquet, Lallemand, Tillequin, Fougeras, & Delaveau 1981; Viscardi, Reynaud, & Reynaud, 1984). The use of a dried flowers' infusion of *C. multiflorus* to control metabolic and endocrine system disorders (such as cholesterol and diabetes), hypertension, rheumatism and headache is widespread in Portugal (Carvalho, 2010; Pardo de Santayana et al., 2007).

### 3.2. Antioxidant activity

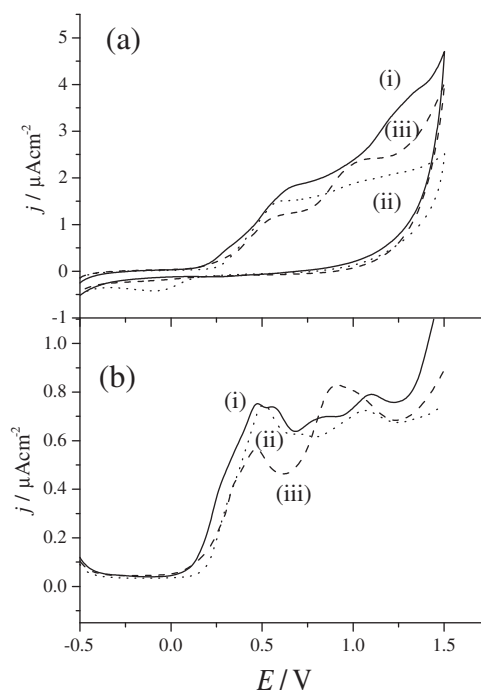
Due to the chemical diversity of antioxidant compounds present in natural samples, it is unrealistic to separate each antioxidant component and study it individually. In addition, levels of single antioxidants do not necessarily reflect their total antioxidant capacity because of the possible synergistic interactions among the antioxidant compounds in a food mixture (Magalhães, Segundo, Reis, & Lima, 2008). Therefore, we evaluated the antioxidant properties of the entire extracts obtained from flowers of the three medicinal plants.

All the samples showed antioxidant activity in the order of *F. ulmaria* > *S. nigra* > *C. multiflorus* (Table 3). Flowers of *F. ulmaria* (meadowsweet) gave the lowest  $EC_{50}$  values in all the assays (lower than 0.1 mg/mL). Particularly for DPPH-scavenging activity assay, this studied sample showed better results (50% at 0.047 mg/mL) than flowering tops of *F. ulmaria* from France (35.0% for water fraction and 59.4% for methanol fraction at 0.1 mg/mL; Calliste et al., 2001). Dawidowicz et al. (2006) reported that flower extracts of *S. nigra* from Poland exhibited higher antioxidant activity (measured by DPPH-neutralising activity and  $\beta$ -carotene-bleaching inhibition assays) than its berries and leaves extracts.

Significantly negative linear correlations were established between the phenolics and flavonoids content, and  $EC_{50}$  values of DPPH-scavenging activity ( $y = -0.0093x + 2.015$ ;  $r^2 = 0.735$  for phenolics and  $y = -0.0327x + 1.933$ ;  $r^2 = 0.794$  for flavonoids;  $p < 0.001$ ), reducing power ( $y = -0.0028x + 0.668$ ;  $r^2 = 0.820$  for phenolics and  $y = -0.0099x + 0.640$ ;  $r^2 = 0.868$  for flavonoids;  $p < 0.001$ ),  $\beta$ -carotene bleaching inhibition ( $y = -0.0012x + 0.353$ ;  $r^2 = 0.740$  for phenolics and  $y = -0.0044x + 0.342$ ;  $r^2 = 0.800$  for flavonoids;  $p < 0.001$ ) and TBARS inhibition ( $y = -0.0012x + 0.297$ ;

$r^2 = 0.715$  for phenolics and  $y = -0.0041x + 0.287$ ;  $r^2 = 0.777$  for flavonoids,  $p < 0.001$ ). This proves that *F. ulmaria* with the highest phenols and flavonoids contents is the most efficient in antioxidant activity (with the lowest  $EC_{50}$  values). The correlations were slightly more significant for flavonoids than for phenolics, and the highest determination coefficients were obtained for reducing power assay.

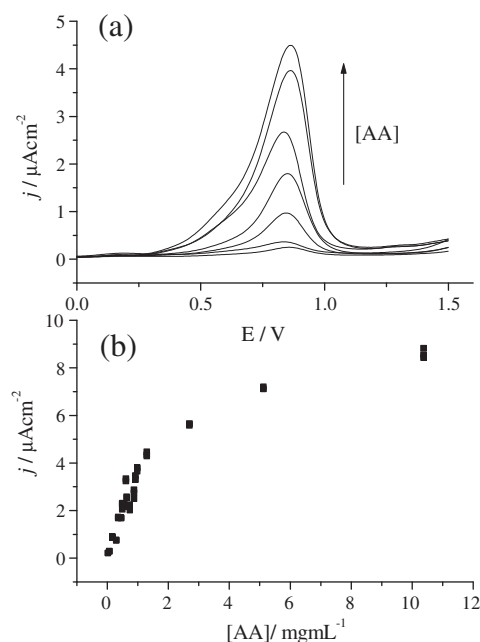
The cyclic voltammograms (CV) observed for the three flower extracts revealed two regions in the potential window under study (Fig. 4a). In the range between  $-0.5$  and  $+0.2$  V, samples were electrochemically silent, with only *S. nigra* showing a low intensity reduction process on the reverse scan at potentials below 0 V. The oxidative current density started then to rise at potentials near 0.2 V, showing maximum intensity around 0.6 V, followed then by a second oxidation process around 1 V. It is also possible to observe some other minor oxidation processes leading to wave broadening or shoulders. This seems to indicate the presence of several electroactive species in the extracts. Peak potentials for the different samples are presented in Table 4. *F. ulmaria* showed a first oxidation process (shoulder) at lower potentials than other samples. This could be explained due to the presence of more easily oxidisable species in this extract. Additionally, the second oxidation process, common to all samples, presents the highest current density in this plant. In fact, *F. ulmaria* showed the highest content in phenolics, flavonoids, total tocopherols and ascorbic acid (Tables 1–3). Moreover, it consistently showed stronger antioxidant activity as measured by DPPH, reducing power,  $\beta$ -carotene bleaching and TBARS methods (Table 3). This is in agreement with the expectation that samples with lower oxidation potentials and higher oxidative current densities should display higher antioxidant activities. Based on CV, the electrochemical behaviour corroborated the greater antioxidant activity found for *F. ulmaria*; however, the comparison for *S. nigra* and *C. multiflorus* is somehow ambiguous since the peak



**Fig. 4.** Electrochemical responses for the sample extracts of (i) *Filipendula ulmaria*, (ii) *Sambucus nigra* and (iii) *Cytisus multiflorus* in methanol/0.1 M NaClO<sub>4</sub> solutions, obtained with a glassy carbon electrode collected between  $-0.5$  and  $1.5$  V (a) Cyclic voltammograms (first scan) at  $100$  mV/s. (b) Differential pulse voltammograms obtained with  $60$  mV pulse amplitude scanned at  $20$  mV/s.

**Table 4**Peak potentials obtained for the electrochemical oxidation processes of the sample extracts (mean  $\pm$  SD;  $n = 6$ ).

	Cyclic voltammetry (V)			Differential pulse voltammetry (V)		
	<i>C. multiflorus</i>	<i>F. ulmaria</i>	<i>S. nigra</i>	<i>C. multiflorus</i>	<i>F. ulmaria</i>	<i>S. nigra</i>
Ep1	–	0.25 $\pm$ 0.03	–	0.35 $\pm$ 0.02	0.28 $\pm$ 0.01	–
Ep2	0.54 $\pm$ 0.01	0.63 $\pm$ 0.01	0.58 $\pm$ 0.00	0.46 $\pm$ 0.01	0.46 $\pm$ 0.00	0.50 $\pm$ 0.00
Ep3	0.99 $\pm$ 0.01	1.23 $\pm$ 0.02	1.01 $\pm$ 0.01	–	0.58 $\pm$ 0.01	–
Ep4	–	–	–	0.91 $\pm$ 0.04	0.82 $\pm$ 0.01	0.71 $\pm$ 0.00
Ep5	–	–	–	1.02 $\pm$ 0.01	1.11 $\pm$ 0.04	1.06 $\pm$ 0.00
Ep6	–	–	–	1.20 $\pm$ 0.00	1.30 $\pm$ 0.01	–

**Fig. 5.** (a) Differential pulse voltammograms obtained for 0.05–1.02 mg/mL ascorbic acid in methanol/0.1 M NaClO<sub>4</sub> solutions, obtained with a glassy carbon electrode between –0.5 and 1.5 V, with 60 mV pulse amplitude at 20 mV/s. (b) Variation of the oxidation peak height, obtained by DPV, with ascorbic acid concentration.

potential is slightly lower for the latter, but *S. nigra* presents a higher current density.

The differential pulse voltammogram (DPV), Fig. 4b, follows a pattern similar to that of CV: a first region between –0.5 and +0.2 V with no significant electrochemical activity, followed by an increase in oxidative current density with maxima around 0.6 and 1 V. In this second region of DPV, however, it was possible to resolve more oxidation processes compared to CV, with *F. ulmaria* displaying at least six distinct oxidation waves, whereas *C. multiflorus* and *S. nigra* showed five and three processes, respectively. Again,

**Table 5**Total electrochemical antioxidant power (TEAP) expressed as ascorbic acid equivalents per gram of sample (DW; mean  $\pm$  SD;  $n = 6$ ), calculated from the DPV experiments.

	Electrochemical antioxidant power (expressed mgAA/g DW)		
	<i>Cytisus multiflorus</i>	<i>Filipendula ulmaria</i>	<i>Sambucus nigra</i>
Ep1	2.19 $\pm$ 0.16	2.71 $\pm$ 0.10	–
Ep2	2.13 $\pm$ 0.65	4.37 $\pm$ 1.02	6.71 $\pm$ 0.53
Ep3	2.81 $\pm$ 0.61	3.43 $\pm$ 0.81	–
Ep4	–	1.60 $\pm$ 0.72	3.48 $\pm$ 0.42
Ep5	2.06 $\pm$ 0.31	1.47 $\pm$ 0.72	2.01 $\pm$ 0.17
Ep6	0.46 $\pm$ 0.00	0.16 $\pm$ 0.09	–
TEAP	9.64 $\pm$ 2.73	13.7 $\pm$ 4.48	12.2 $\pm$ 1.65

*F. ulmaria* showed a rise in current density at lower potentials than other samples. In terms of intensity it is *C. multiflorus* that shows the lowest current density for the common oxidation process at 0.5 V, with the other two samples presenting a similar maximum value. This profile is not standard for the entire voltammogram, with the oxidative current density of *C. multiflorus* surpassing that of *F. ulmaria* at potentials around 0.9 V.

In order to quantify the electrochemical antioxidant activity of samples we compared the current density of all oxidation peaks (peak height) with that of ascorbic acid (AA), which shows an irreversible oxidation peak around 0.86 V in methanol solutions containing 0.1 M NaClO<sub>4</sub>. Fig. 5a and b show the DPV voltammograms and the variation of peak current density plotted against AA concentration, respectively, where a linear correlation was found in the range 0.05–1.02 mg/mL. This allows the expression of the contribution of each oxidation process to the electrochemical antioxidant activity in terms of equivalents of ascorbic acid.

Table 5 presents the quantitative results for all the resolved oxidation peaks obtained from DPV for the three samples under study. *F. ulmaria* shows, at lower oxidation potential, the most intense antioxidant processes; however, we cannot exclude the possible contribution of other oxidation process to the antioxidant activity. In fact, the capability for the sample to act as oxidative protector arises from the existence of easily oxidised species (low oxidation potential) and their amount, as well as from the presence of other less oxidisable species, providing that the substance to be protected has a higher oxidation potential. To account for the contributions of all species, we expressed the sum of AA equivalents as total electrochemical antioxidant power, TEAP. The calculated TEAP values were significantly lower for *C. multiflorus*, as found with the other antioxidant assays, but similar for *F. ulmaria* and *S. nigra*, suggesting that, for these species, the presence of the easily oxidised species mentioned above could make the difference.

#### 4. Conclusions

Flowers of *F. ulmaria*, *S. nigra* and *C. multiflorus* were analysed for their phytochemical composition, and antioxidant activities were evaluated based of chemical, biochemical and electrochemical assays. The overall antioxidant activity of samples was found to vary in order *F. ulmaria* > *S. nigra* > *C. multiflorus*, irrespective of the method employed. These results were found to be in agreement with their content in antioxidants, especially total phenolics, flavonoids and ascorbic acid. This suggests that the electrochemical methods such as CV and DPV employed in this study are suitable for fast and inexpensive screening, profiling and quantification of antioxidant activity in complex plant matrices. These methods may become portable and enable rapid in-field analysis. Moreover, they are free from laborious sample preparation, use a minimum of reagents because they are based on an intrinsic property of the analyte (electroactive) and are applicable to non-transparent samples (Blasco et al., 2005).

These results also indicate there may be a basis for the ethnopharmacological use of these wild medicinal plants, related to their



antioxidant and phytochemical composition. As such, that the studied extracts could be suitable for incorporation into functional beverages or products with potential anti-inflammatory and other health-promoting properties related to oxidative stress.

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