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 since a long time ago, 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 (2696 μg/g dw) and tocopherols (497 μg/g dw). Accordingly, the antioxidant activity was found to vary in order *F. ulmaria* > *S. nigra* > *C. multiflorus* irrespective of the analysis method. Electrochemical methods have proven to be expedite and inexpensive techniques to characterize the antioxidant activity of plant extracts. The studied wild medicinal flowers could be selected for processing extracts with health-promoting properties or to be incorporate into functional beverages or products with potential anti-inflammatory and other properties related to oxidative stress.

***Keywords**: Cytisus multiflorus*; *Filipendula ulmaria*; *Sambucus nigra*; Antioxidants; Phytochemicals; Chromatography; Electrochemistry

1. Introduction

Reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide, hydroxyl, acyl and alkyloxy 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 aging process itself (Halliwell, 1996; Gutteridge & Halliwell, 2000; Valko et al., 2007). Natural antioxidants protect the human body from free radicals, prevent oxidative stress and associated diseases (López et al., 2007; Ferreira, Barros, & Abreu, 2009). 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 for accurate and reliable information on their phytochemicals and antioxidant activity, as well as on the potential benefits and prospective products, such as nutraceutics 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), both 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 recognized 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 syrup (Camejo-Rodrigues, Ascensão, Bonet, & Vallès, 2003; Carvalho, 2010; Pardo de Santayana et al., 2007; Pardo de Santayana, 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 none or very little side effects are desirable to substitute 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 (Prior, Wu, & Schaich, 2005; Frankel & Finley, 2008). With respect to electrochemical methods, there is considerable work done on characterizing the behavior of isolated natural products. Voltammetric in particular 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 as 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; Litescu & Radu, 2000; Kilmartin, Zou, & Waterhouse, 2001).

Herein the antioxidant capacity of these three wild medicinal plants was evaluated by radical scavenging activity and lipid peroxidation inhibition *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 (Blasco, Rogerio, González, & Escarpa, 2005; Cosio, Buratti, Mannino, & Benedetti, 2006; Barros et al., 2008). The aim of this study was to obtain a complete characterization 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 with flowers fully open and functional of the three species 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 are deposited in the Herbário da Escola Superior Agrária de Bragança (BRESA). Samples were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at -20ºC 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 acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also 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 (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Sodium perchlorate monohydrate dihydrate, purity grade, was purchased from Fluka, and was dried overnight at 30ºC 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, USA).

2.3. Phytochemicals and antioxidants

2.3.1. Determination of tocopherols

BHT solution in hexane (10 mg/mL; 100 μl) and tocol solution in hexane (internal standard- IS); 50 μg/mL; 400 μl) were added to the sample prior to the extraction procedure. The samples (~500 mg) were homogenized 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 homogenized (1 min), centrifuged (Centorion K24OR- 2003 refrigerated centrifuge; 5 min, 4000g) 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 µm 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), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) 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). The chromatographic separation was achieved with a Polyamide II (250 x 4.6 mm) normal-phase column from YMC Waters operating at 30ºC. 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 µl. 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 µg 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 Nº 4 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 (Analytikijena 200-2004 spectrophotometer). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (6.0×10^{-3} -1.0×10^{-1} mg/mL), and the results were expressed as mg per 100 g of dry weight.

2.3.3. Determination of lipo-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 β-carotene was calculated according to the following equation: β-carotene (mg/100 mL) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; Lycopene (mg/100 mL) = $-0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; Chlorophyll a (mg/100 mL) = $0.999 \times A_{663} - 0.0989 \times A_{645}$; Chlorophyll b (mg/100 mL) = $-0.328 \times A_{663} + 1.77 \times A_{645}$, and further expressed in µg per g of dry weight (dw).

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 ºC for 30 min. The resulting suspension was centrifuged at 15,000 g for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure (rotary evaporator Büchi R-210) and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters from Whatman. 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 NH_2 column (4.6 x 250 mm, 5 µm, Knauer) operating at 30ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized 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 normalization of the chromatographic peak area and the results are expressed in mg per g of dry weight (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), during at least 12 h in a bath at 50 ºC 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 vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 µm nylon filter from Whatman. The fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m x 0.32 mm ID x 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 10ºC/min ramp to 240 ºC and held for 11 min. The carrier gas (Hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. For each analysis 1 µl of the sample was injected in 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; ~1g) was stirred with 50 mL of methanol at 25 ºC at 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 35ºC under reduced pressure, re-dissolved in methanol at 20 mg/mL (stock solution), and stored at 4 ºC 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 (9.4×10^{-3}-1.5×10^{-1} mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (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 $NaNO_2$ solution (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand 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 (4.5×10^{-3} - 2.9×10^{-1} mg/mL) and the results were expressed as mg of (+)-catechin equivalents (CE) per g of dry weight (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 (Bio-Tek Instruments, Inc) consisted of one of the different concentrations of the extracts (0.03–1.00 mg/mL; 30 μl) and aqueous methanolic solution (80:20 v/v, 270 μl) containing DPPH radicals ($6x10^{-5}$ mol/L). 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: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

2.4.3. 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 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was transferred to 48-wells 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.

2.4.4. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40ºC 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 50ºC 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 β-carotene, was prepared for background subtraction. β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

2.4.5. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) 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 $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37ºC 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 ºC 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: Inhibition ratio (%) = [(A – B)/A] x 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_{50}) 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 fine lyophilized powder of plant flowers (20 mesh; 1g) was extracted with 50 mL methanol in the refrigerator (~4 ºC) 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 $NaClO_4$ so that the final concentration of support electrolyte was 0.1M. Solutions were immediately analyzed.

2.5.2. Instrumentation

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on an Autolab PGSTAT 302 potentiostat/galvanostat using a closed standard three electrode cell. A glassy carbon electrode (MF-2012, ϕ = 3.0 mm, BAS Inc.) was used as working electrode and a Pt foil as counter electrode. All potentials

refer to an Ag/AgCl 3 M KCl (+207mV *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 (Beuhler) polishing pad wetted with an aqueous suspension of 0.3 μm alumina (Beuhler), ii) sonicated in HCl 6 M during 1 minute, and then in methanol, and iii) submitted to an anodic current at 2.0V for 60s. The electrode was thoroughly rinsed with deionised water and methanol between every step, and dried blotted. 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 compound were studied in methanol 0.1 M $NaClO_4$. All the solutions were analysed immediately after preparation and the electrochemical responses recorded after immersion of the glassy carbon electrode, to minimize adsorption of species onto the electrode surface prior to run. Cyclic voltammetry (CV) was used to characterize 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 and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 16.0 programm.

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 (> 1729 µg/g dw) and α-tocopherol was the major tocopherol in all the samples (> 313 µg/g); *F. ulmaria* was the richest sample in α-tocopherol; its tocopherols profile is shown in **Figure 1**. δ-Tocopherol was not detected in *S. nigra* and was the minor compound in the other two plants. Flowers of *F. ulmaria* and *C. multiflorus* presented the highest content of ascorbic acid (2696.26 µg/g and 2674.78 µg/g, respectively) and tocopherols (496.61 µg/g and 482.77 µg/g, respectively), without significant differences, $p<0.05$, between them. Kaack & Austed

(1998) reported the quantification of ascorbic acid in fresh fruits of *S. nigra* (60 to 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 oxidized vitamin E. The interactions 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 β-carotene (53.85 µg/g dw), lycopene (11.28 µg/g) and chlorophyll b (51.61 µg/g) were higher in *C. multiflorus* flowers. *F. ulmaria* revealed the highest levels of chlorophyll a (78.15 µg/g). Carotenoids are amongst nature's most widespread pigments and have also received substantial attention because of both their provitamin and antioxidant roles. The peroxyl radicals ($ROO^{\bullet}$) formed from lipids (especially polyunsaturated phospholipids) are very damaging to cells. The extensive systems of double bonds make carotenoids susceptible to attack by peroxyl 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 presented 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 (137.94 mg/g dw), with the highest levels of glucose (42.73 mg/g) and fructose (69.27 mg/g), both of them reducing sugars. The sugars profile of this sample is shown in **Figure 2**. Veberic et al. (2009) reported the concentrations of individual sugars in fruits of *S. nigra* (68.53 to 104.16 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 α-linolenic acid (C18:3n3; 25 to 33%) followed by linoleic acid (C18:2n6; 18 to 25%) contributing to the prevalence of PUFA in all the samples. Some authors showed that linoleic acid

(C18:2n6), 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 to 18%). This fatty acid was the major compound found in pollen of *S. nigra* (35.69%), followed by C18:1n9 (14.26%), C18:2n6 (12.79%) and C18:3n3 (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 **Figure 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 (45.62-92.73 mg GAE/g dw; **Table 3**); *Filipendula 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, Kapsokefalou, & 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 to 119 mg/g) (Harbourne, Marete et al., 2009), temperatures (39 to 63 mg/g) and pH (43 to 57 mg/g) (Harbourne, Jacquier et al., 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) (Scheer & Wichtl, 1987; Calliste, Trouillas, Allais, Simon, & Duroux, 2001; Fecka, 2009; Harbourne, Marete, Jacquier, & O'Riordan, 2009; Harbourne, Jacquier, & O'Riordan, 2009). 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 their 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 anticancerogenic and antioxidative effect of elderberry juice was also 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 consumption of an elderberry juice induces a significant rise in plasma antioxidant capacity and a significant decrease in plasma malondialdehyde *in vivo* (Netzel et al., 2002; Netzel et al., 2005).

Flowers of *S. nigra* have been found to contain hydroxycinamic acids, mono- and di-caffeoylquinic acid derivatives, flavones and flavonol glycosides (Dawidowicz, Wianowska, Gawdzik, & Smolarz, 2003; Christensen, Kaack, & Fretté, 2008; Rieder, 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). Their 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, Jakopic, Stampar, & Schmitzer, 2009). Flu and other health related remedies were developed using elderflowers (Kaack & Austed, 1998). Different European ethnobotanical surveys document that elder can satisfy several traditional needs being the medicinal one of the most important (Vallès, Bonet, & Agelet, 2004; Carvalho, 2010; Neves, Matosa, Moutinho, Queiroz, & Gomes, 2009; Parada, Carrió, Bonet, & Vallès, 2009). 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-acetyl scoparin), flavonol glycosides (rutin, quercitin, kaempferol, isorhamnetin, quercitrin) and isoflavones (genistein, sarothamnoside) (Brum-Bousquet, Lallemand, Tillequin, Fougeras, & Delaveau 1981; Viscardi, Reynaud, & Raynaud,1984). The use *Cytisus multiflorus* dried flowers' infusion 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 neutralizing activity and β-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 ($y = –0.0093x + 2.0152$; R^2 0.7348 for phenolics and $y = –0.0327x + 1.9331$; R^2 0.7941 for flavonoids; $p<0.001$), reducing power ($y = –0.0028x + 0.6681$; R^2 0.8197 for phenolics and y =

–0.0099x + 0.6398; R^2 0.8681for flavonoids; p<0.001), β-carotene bleaching inhibition (y = –0.0012x + 0.3529; R^2 0.7395 for phenolics and y = –0.0044x + 0.3419; R^2 0.8004 for flavonoids, p<0.001) and TBARS inhibition (y = –0.0012x + 0.2971; R^2 0.7145 for phenolics and y = –0.0041x + 0.2874; R^2 0.7771 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, **Figure 4a**. In the range between -0.5 and +0.2V samples were electrochemically silent, with only *S. nigra* showing a low intensity reduction process on the reverse scan at potentials below 0V. 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 1V. 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 oxidizable 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, 2** and **3**). Moreover, it consistently showed stronger antioxidant activity as measured by DPPH, reducing

power, β-carotene bleaching and TBARS methods (**Table 3**). This is in agreement with the expectation that samples with lower oxidation potentials and higher oxidative currents 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 potential is slightly lower for the latter, but *S. nigra* present a higher current density.

The differential pulse voltammogram (DPV), **Figure 4b**, follows a pattern similar to that of CV: a first region between -0.5 and +0.2V with no significant electrochemical activity, followed by an increase in oxidative current density with maxima around 0.6 and 1V. In this second region of DPV, however, it was possible to resolve more oxidation processes compared to CV, with *F. ulmaria* displaying at least 6 distinct oxidation waves, whereas *C. multiflorus* and *S. nigra* showed 5 and 3 processes, respectively. Again, *F. ulmaria* showed a rise in current density at lower potentials than other samples, with *S. nigra* being the last. In terms of intensity it is *C. multiflorus* that show 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 voltamogram, with the oxidative current density of *C. multiflorus* surpassed that of *F. ulmaria* at potentials around 0.9V.

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 0.86V in methanol solutions containing 0.1 M $NaClO_4$. **Figures 5a** and **5b** 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 to 1.02 mg/mL. This allows expressing 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 oxidise species (low oxidation potential) and their amount, but as well from the presence of other less oxidisable species, providing that the substance to be protect have an higher oxidation potential. To account 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 this species, the presence of the easily oxidise 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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Table 1. Composition of the 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	*Cytisus multiflorus*	*Filipendula ulmaria*	*Sambucus nigra*
α-tocopherol	347.67 ± 17.51 b	425.09 ± 15.45 a	313.30 ± 4.08 c
β-tocopherol	34.82 ± 4.14 a	15.91 ± 0.13 b	8.12 ± 0.71 c
γ-tocopherol	92.64 ± 4.51 a	44.32 ± 1.66 b	2.89 ± 0.18 c
δ-tocopherol	7.63 ± 0.46 b	11.29 ± 0.92 a	nd
Total tocopherols	482.77 ± 9.32 a	496.61 ± 16.06 a	324.31 ± 4.61 b
Ascorbic acid	2674.78 ± 12.57 a	2696.26 ± 27.02 a	1729.41 ± 4.79 b
β-carotene	53.85 ± 0.06 a	36.32 ± 0.17 b	18.43 ± 0.05 c
Lycopene	11.28 ± 0.04 a	6.18 ± 0.08 b	5.34 ± 0.04 c
Chlorophyll a	60.32 ± 0.05 b	78.15 ± 0.07 a	44.97 ± 0.02 c
Chlorophyll b	51.61 ± 0.10 a	46.73 ± 0.20 b	31.99 ± 0.06 c
Glucose	42.73 ± 1.40 a	13.09 ± 0.61 c	32.28 ± 0.66 b
Fructose	69.27 ± 2.71 a	19.84 ± 0.65 c	26.61 ± 0.96 b
Sucrose	18.32 ± 0.73 b	11.34 ± 0.44 c	24.71 ± 0.26 a
Trehalose	7.62 ± 0.98 b	20.85 ± 1.17 a	nd
Total sugars	137.94 ± 5.82 a	65.12 ± 0.53 c	83.61 ± 0.92 b

nd- not detected

Table 2. Composition (%) of the medicinal plants in fatty acids (mean ± SD; n=9). In each column different letters mean significant differences ($p<0.05$).

Fatty acid	*Cytisus multiflorus*	*Filipendula ulmaria*	*Sambucus nigra*
C6:0	0.10 ± 0.01	2.16 ± 0.11	0.03 ± 0.00
C8:0	0.06 ± 0.00	0.19 ± 0.01	0.02 ± 0.00
C10:0	0.04 ± 0.00	0.06 ± 0.00	0.02 ± 0.00
C12:0	0.33 ± 0.00	0.73 ± 0.01	0.14 ± 0.00
C14:0	0.53 ± 0.02	10.05 ± 0.53	0.42 ± 0.03
C14:1	0.05 ± 0.00	0.11 ± 0.01	0.05 ± 0.00
C15:0	0.13 ± 0.01	0.12 ± 0.00	0.15 ± 0.00
C15:1	nd	nd	2.07 ± 0.41
C16:0	11.14 ± 0.02	12.22 ± 0.03	18.06 ± 0.53
C16:1	0.60 ± 0.02	Nd	0.36 ± 0.04
C17:0	0.37 ± 0.02	0.37 ± 0.03	0.28 ± 0.02
C18:0	4.09 ± 0.02	3.93 ± 0.13	2.02 ± 0.17
C18:1n9c	6.59 ± 0.76	2.28 ± 0.10	11.93 ± 0.16
C18:2n6c	24.31 ± 0.28	18.02 ± 0.58	24.64 ± 0.49
C18:3n6	nd	0.11 ± 0.01	nd
C18:3n3	32.49 ± 1.11	32.89 ± 0.47	25.43 ± 1.73
C20:0	3.21 ± 0.08	3.48 ± 0.25	1.26 ± 0.10
C20:1c	0.47 ± 0.02	0.32 ± 0.05	0.81 ± 0.02
C20:2c	0.18 ± 0.02	0.06 ± 0.00	nd
C20:3n3+C21:0	0.36 ± 0.01	1.10 ± 0.05	0.21 ±0.01
C20:5n3	nd	0.85 ± 0.05	0.57 ± 0.03
C22:0	1.68 ± 0.05	2.53 ± 0.09	1.99 ± 0.20
C23:0	11.26 ± 0.29	4.35 ± 0.01	1.81 ± 0.26
C24:0	2.07 ± 0.04	4.06 ± 0.14	7.74 ± 1.21
Total SFA	34.98 ± 0.33 b	44.26 ± 0.67 a	33.92 ± 1.47 b
Total MUFA	7.71 ± 1.75 b	2.71 ± 0.16 c	15.23 ± 0.64 a
Total PUFA	57.34 ± 1.38 a	53.03 ± 0.83 b	50.85 ± 2.11 b

nd- not detected; Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric

acid (C12:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); *cis*-10-pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1c); *cis*-11,14-Eicosadienoic acid (C20:2c); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); Eicosapentacenoic acid (C20:5n3); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).

Table 3. Extraction yields, content in phenolics and flavonoids, and antioxidant activity (EC_{50} values, mg/mL) of the medicinal plants (mean ± SD; n=9). In each row different letters mean significant differences ($p<0.05$).

	Cytisus multiflorus	*Filipendula ulmaria*	*Sambucus nigra*
Extraction yield (%)	36.30 ± 3.27 a	29.30 ± 2.03 b	37.74 ± 2.12 a
Phenolics (mg GAE/g dw)	45.62 ± 3.29 c	227.60 ± 0.53 a	92.73 ± 4.66 b
Flavonoids (mg CE/g dw)	7.86 ± 0.42 c	62.40 ± 4.83 a	26.18 ± 0.51 b
DPPH scavenging activity	2.02 ± 0.11 a	0.05 ± 0.01 c	0.57 ± 0.03 b
Reducing power	0.64 ± 0.02 a	0.06 ± 0.00 c	0.27 ± 0.01 b
β-carotene bleaching inhibition	0.35 ± 0.01 a	0.09 ± 0.01 c	0.16 ± 0.01 b
TBARS inhibition	0.30 ± 0.02 a	0.05 ± 0.00 c	0.12 ± 0.01 b

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

	Cyclic Voltammetry (V)			Differential Pulse Voltammetry (V)		
	C. multiflorus	*F. ulmaria*	*S. nigra*	*C. multiflorus*	*F. ulmaria*	*S. nigra*
Ep1	-	0.25 ±0.03	-	0.35 ±0.02	0.28 ±0.01	-
Ep2	0.54 ±0.01	0.63 ±0.01	0.58 ±0.00	0.46 ±0.01	0.46 ±0.00	0.50 ±0.00
Ep3	0.99 ±0.01	1.23 ±0.02	1.01 ±0.01	-	0.58 ±0.01	-
Ep4	-	-	-	0.91 ±0.04	0.82 ±0.01	0.71 ±0.00
Ep5	-	-	-	1.02 ±0.01	1.11 ±0.04	1.06 ±0.00
Ep6	-	-	-	1.20 ±0.00	1.30 ±0.01	-

Table 5. Total Electrochemical Antioxidant Power (TEAP) expressed as ascorbic acid equivalents per gram of sample (dw; mean ± SD; n=6), calculated from the DPV experiments.

	Electrochemical antioxidant power (expressed mgAA/g dw)		
	Cytisus multiflorus	*Filipendula ulmaria*	*Sambucus nigra*
Ep1	2.19 ±0.16	2.71 ±0.10	-
Ep2	2.13 ±0.65	4.37 ±1.02	6.71 ±0.53
Ep3	2.81 ±0.61	3.43 ±0.81	-
Ep4	-	1.60 ±0.72	3.48 ±0.42
Ep5	2.06 ±0.31	1.47 ±0.72	2.01 ±0.17
Ep6	0.46 ± 0.00	0.16 ±0.09	-
TEAP	9.64 ±2.73	13.74 ±4.48	12.19 ±1.65

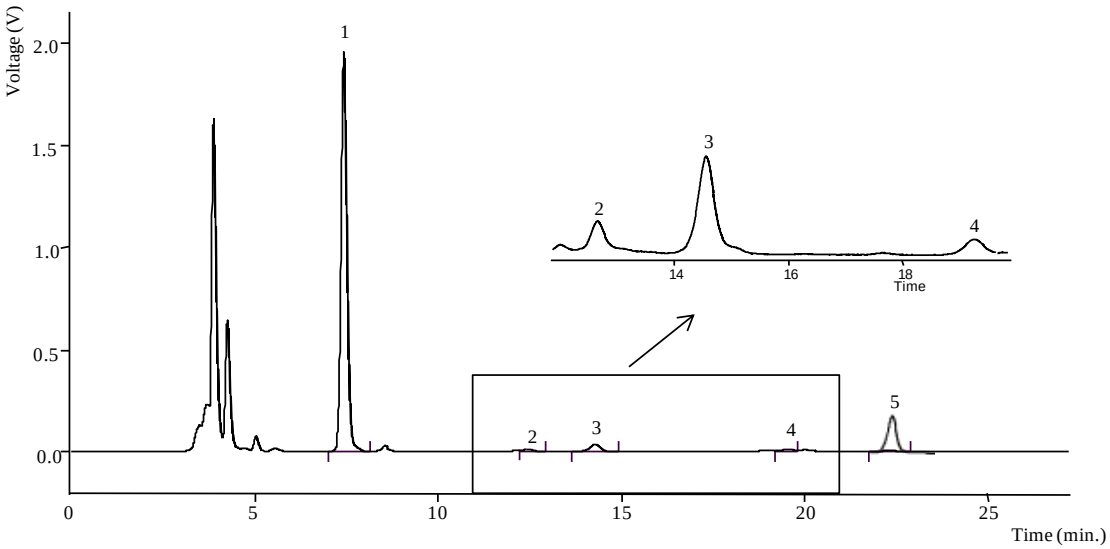

Figure 1. Tocoferols profile of *F. ulmaria*. 1-α-Tocoferol; 2-β-tocoferol; 3-γ- tocoferol; 4- δ-tocoferol; 5-tocol (IS).

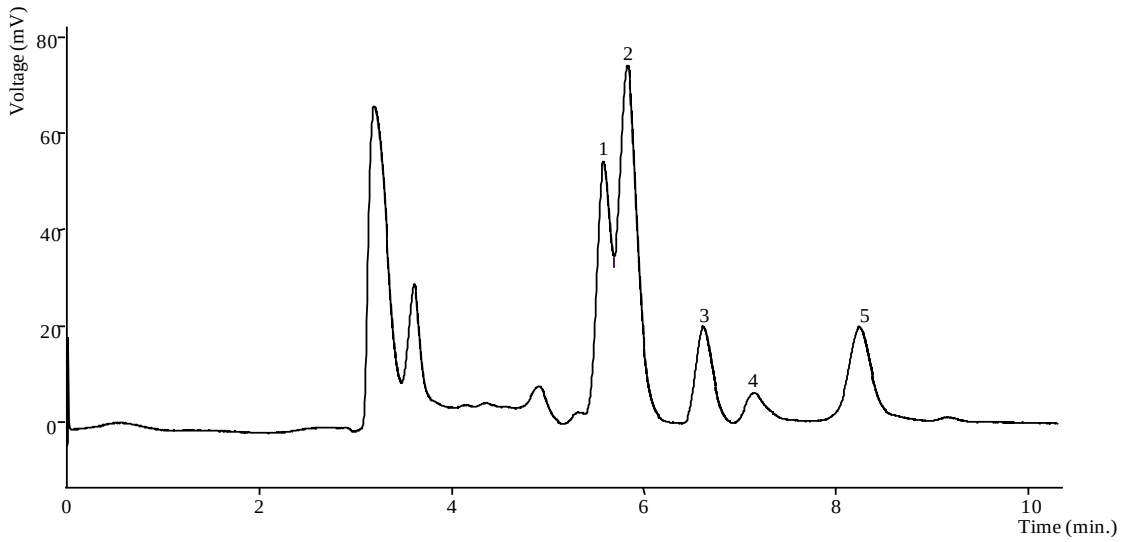

Figure 2. Sugars profile of *C. multiflorus*. 1-Fructose; 2-glucose; 3-sucrose; 4-trehalose; 5-melezitose (IS).

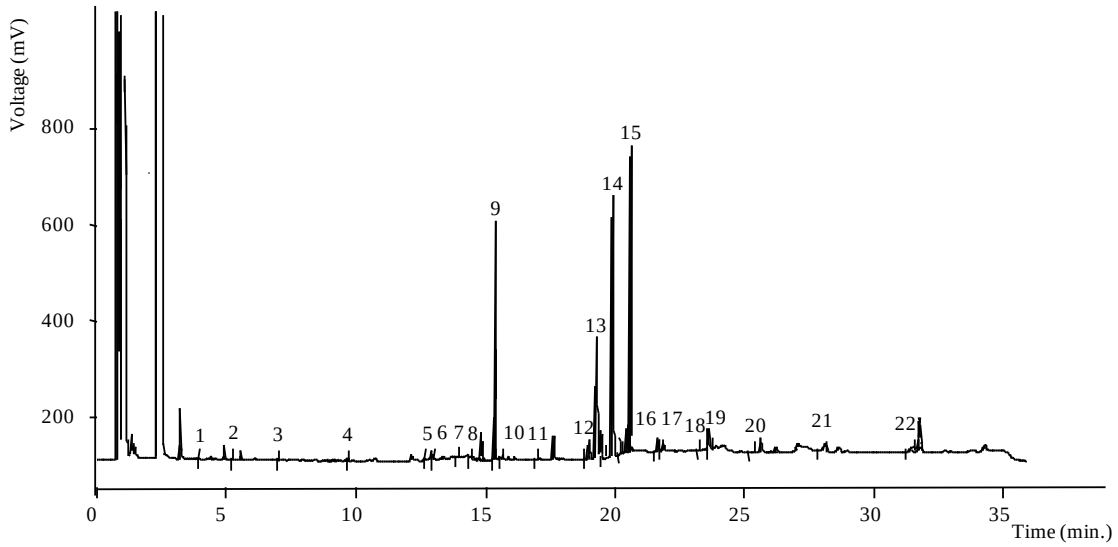

Figure 3. Fatty acids profile of *S. nigra*. 1- C6:0; 2- C8:0; 3- C10:0; 4- C12:0; 5- C14:0; 6-C14:1; 7- C15:0; 8- C15:1; 9- C16:0; 10- C16:1; 11- C17:0; 12- C18:0; 13- C18:1n9; 14 C18:2n6; 15- C18:3n3; 16- C20:0; 17- C20:1; 18- C20:3n3+C21:0; 19- C20:5n3; 20- C22:0; 21- C23:0; 22- C24:0.

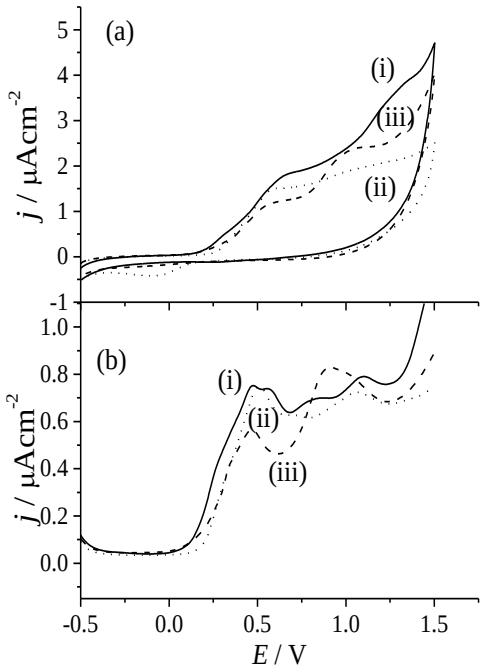

Figure 4. Electrochemical responses for the sample extracts of (i) *Filipendula ulmaria*,

(ii) *Sambucus nigra* and (iii) *Cytisus multiflorus* in methanol/$NaClO_4$ 0.1M solutions, obtained with a glassy carbon electrode collected between -0.5 and 1.5 V (a) Cyclic voltammograms (first scan) at 100 $mV.s^{-1}$. (b) Differential pulse voltammograms obtained with 60 mV pulse amplitude scanned at 20 $mV.s^{-1}$.

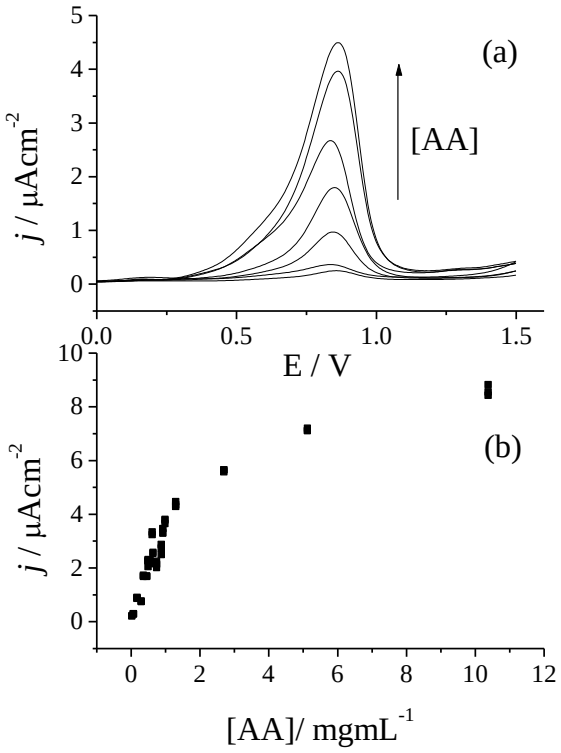

Figure 5. (a) Differential pulse voltammograms obtained for 0.05 to 1.02 $mgmL^{-1}$ ascorbic acid in methanol/$NaClO_4$ 0.1M solutions, obtained with a glassy carbon electrode between -0.5 and 1.5 V, with 60 mV pulse amplitude at 20 $mV.s^{-1}$. (b) Variation of the oxidation peak height, obtained by DPV, with ascorbic acid concentration.