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Enhancement of nutritional and bioactive compounds by *in vitro* culture of wild *Fragaria vesca* L. vegetative parts

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ABSTRACT

In vitro culture emerges as a sustainable way to produce bioactives for further applicability in the food industry. Herein, vegetative parts of *Fragaria vesca* L. (wild strawberry) obtained by *in vitro* culture were analyzed regarding nutritional and phytochemical compounds, as well as antioxidant activity. These samples proved to have higher content of protein, polyunsaturated fatty acids, soluble sugars, organic acids (including ascorbic acid) and tocopherols (mainly α-tocopherol) than wild grown *F. vesca*, as well as containing additional phenolic compounds. The antioxidant activity of hydromethanolic extracts could be correlated with the content of different phenolic groups and other compounds (sugars and organic acids). It was demonstrated that *in vitro* culture could enhance nutritional and bioactive compounds of *Fragaria vesca* L. plants, providing a very interesting biotechnological tool for potential food applications.

1. Introduction

Wild strawberry (*Fragaria vesca* L., Rosaceae family) can be commonly found in Europe, Japan, North America and Canada, growing wild in mountain zones, forests, on slopes and roadsides (Castroviejo et al., 1998). The plant is mainly appreciated for its sweet small fruits; however the vegetative parts have been described as important sources of macro and micronutrients and also phenolic compounds (procyanidins, ellagic acid and hydroxycinnamoyl derivatives) with strong antioxidant activity (Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Morales, et al., 2015). Vegetative parts from *F. vesca* could provide tonic, antiseptic and detoxifying properties. Furthermore, its decoctions and infusions have been traditionally used to treat urinary tract infections and hypertension, presenting also antidiarrheal and anticoagulant activity (Camejo-Rodrigues, Ascensão, Bonet, & Vallès, 2003; Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska, & Gancarz, 2009; Savo, Giulia, Maria, & David, 2011; Özüdoru, Akaydin, Erik, & Yesilada, 2011).

The growing demand for natural products that complement their nutritional role with additional functional properties requires innovation in the ways to obtain these products, in order to protect wild populations from where they are obtained, and also to avoid competing directly with crops that are used for food (Godfray et al., 2012). Plant tissue culture appears as a valuable technique to produce secondary metabolites, being an ecological and sustainable alternative for the production of endangered species (by overexploitation), as well as a means to obtain bioactive extracts and compounds that can be further applied in pharmaceutical/medical fields or in the food industry. Indeed, this approach has been endorsed by FAO as a safe process to produce compounds for food applications (Dias, Sousa, Alves, & Ferreira, 2016). Regardless of climate or geographical conditions, this technique allows a continuous production of natural compounds under a very restricted controlled regime (Anand, 2010; Karuppusamy, 2009).

The nutritional value and chemical profile of vegetative parts of *F. vesca* were previously reported by our research group (Dias, Barros, Morales, et al., 2015). The presence of sugars and organic acids was also described in its fruits (Doumett et al., 2011; Ornelas-Paz et al., 2013), while phenolic compounds and related bioactive properties were reported in different plant parts (fruits, leaves and roots) (Dias et al., 2016; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, Santos-Buelga, & Ferreira, 2015c).

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These compounds were also described in *F. vesca* obtained from *in vitro* culture, after optimization of growth conditions (concentration of plant regulators and regeneration enhancers) (Yildirim & Turker, 2014). Nevertheless and to the author's best knowledge, no other components have been studied.

In the present work, vegetative parts of *Fragaria vesca* L. were obtained by *in vitro* culture and further characterized in terms of macronutrients, fatty acids, soluble sugars, organic acids, tocopherols and phenolic compounds; their antioxidant activities were also studied. The studies were carried out with lyophilized material, hydromethanolic extracts and aqueous consumption forms (infusions and decoctions).

2. Materials and methods

2.1. Standards and reagents

Acetonitrile (99.9%), *n*-hexane (95%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Acetonitrile (fluorescence grade) was bought from Fisher Scientific (Madrid, Spain). Formic acid was purchased from Prolabo (VWR International, France). Fatty acids methyl ester (FAME) reference standard mixture (standard 47885-U) was purchased from Sigma (St. Louis, MO), alongside other individual fatty acid methyl ester isomers, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-car boxylic acid), l-ascorbic acid, tocopherol, sugar and organic acid standards. Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and establishment of an in vitro culture of Fragaria vesca L

The samples of wild *Fragaria vesca* L. fruits were collected in Serra da Nogueira, Bragança, north-eastern Portugal, in July 2013. The establishment of the *in vitro* culture was achieved by using the wild fruits with seeds. The fruits were washed with tap water and sterilized with bleach and detergent for 5 min under agitation, washed with sterilized water and inoculated in a basic medium for seed germination with water and agar (0.9%) and kept in the dark until germination. The seedlings were then detached from the fruit and placed in a modified culture medium (Murashige & Skoog, 1962) supplied with macronutrients, l mg/L thiamine, 1 mg/L nicotinic acid, 1 mg/L pyridoxine, 2% sucrose, 0.5 mg/L BAP (benzylaminopurine) and 0.5 mg/L IBA (indole-3-butyric acid). The pH culture medium was adjusted to 5.7 before autoclaving. The culture conditions were Tmin [16–18] °C, Tmax [24–26] °C with a photoperiod of 16/8 h (light/dark) supplied by light bulbs (Silvana day light; Philips, Eindhoven, Netherlands). The plants were kept under the same culture conditions and subcultured every month, collecting the aerial parts obtained under *in vitro* conditions and keeping the roots and shoots for further growth and collection of new material. The collected aerial parts were stored at −20 °C, lyophilized and reduced to a fine powder for further analysis.

2.3. Preparation of the aqueous consumption forms

For infusions preparation, the lyophilized plant material (500 mg) was added to 100 mL of boiling distilled water (pH 6.6) at 100 °C, left to stand at room temperature for 5 min and then filtered (0.22 μm) under reduced pressure.

For decoctions preparation, the lyophilized plant material (500 mg) was added to 100 mL of distilled water, heated and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The extracts obtained by infusion and decoction were lyophilized for further analysis of phenolic compounds and antioxidant activity.

2.4. Nutritional value of the lyophilized plant material

2.4.1. Proximate composition

The lyophilized plant material was analyzed for proteins, fat, carbohydrates and ash according to AOAC procedures (AOAC, 2005). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method (AOAC, 991.02); the crude fat (AOAC, 989.05) was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content (AOAC, 935.42) was determined by incineration at 550 ± 15 °C; Total carbohydrates were calculated by difference. Total energy was calculated according to the following equation:

$$\text{Energy}(\text{kcal}/100\text{g}) = 4 \times (\text{g proteins} + \text{g carbohydrates}) + 9 \times (\text{g fat}),$$

according to the (Regulation (EC) No 1169/2011, 2011).

2.4.2. Fatty acids

Fatty acids were determined in the lyophilized plant material, after a transesterification process, as previously described (Barros et al., 2013). The fatty acids profile was analyzed using a gas chromatograph (model GC 1000 instrument; DANI, Contone, Switzerland) equipped with a split/splitless injector and a flame ionization detector (GC-FID, 260 °C) and a Macherey-Nagel (Düren, Germany) column (0.5 g/kg cyanopropyl-methyl-0.5 g/kg phenyl-methylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm film thickness). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (61000 Pa), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Identifications were made by comparing the relative retention times of FAME (fatty acid methyl esters) peaks of the samples with commercial standards. The results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Prague, Czech Republic) and expressed as a relative percentage of each fatty acid.

2.5. Chemical characterization of the lyophilized plant material and aqueous consumption forms

2.5.1. Soluble sugars

The extraction of soluble sugars from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly. Soluble sugars were determined by high-performance liquid chromatography equipment consisting of an integrated system with a pump (Smartline system 1000; Knauer, Berlin, Germany), degasser system (Smartline Mmanager 5000) and autosampler (AS-2057; Jasco, Easton, MD), coupled to a refractive index detector (HPLC-RI; Smartline system 1000; Knauer, Berlin, Germany), as previously described (Barros et al., 2013). The chromatographic separation was achieved with a Eurospher 100–5 NH_2 column (5 μm, 250 × 4.6 mm i.d., Knauer) operating at 35 °C. The mobile phase was acetonitrile:deionized water (7:3, *v/v*), at a flow rate of 1 mL/min. The identification was carried out by chromatographic comparisons of the relative retention times of sample peaks with authentic standards, while the quan-

tification was performed using the internal standard (melezitose) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g dry weight or in mg per 100 mL in the case of infusions and decoctions.

2.5.2. Organic acids

The extraction of organic acids from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly. Vitamin C and other organic acids were determined by ultra-fast liquid chromatography coupled to photodiode array detection (UFLC-PDA; Shimadzu Corporation, Kyoto, Japan) and following a procedure previously described (Barros et al., 2013). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA) reverse phase C_{18} column (5 μm, 250 × 4.6 mm) thermostatted at 35 °C. The elution was performed with sulfuric acid (3.6 mmol/L) using a flow rate of 0.8 mL/min. The quantification was performed by comparison of the area of the peaks recorded at 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight or in mg per 100 mL in the case of infusions and decoctions.

2.5.3. Tocopherols

The extraction of tocopherols from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly using HPLC coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (5 μm, 250 × 4.6 mm i.d.; YMC, Waters Corporation, Milford, MA), operating at 35 °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. The identification was performed by chromatographic comparisons with authentic standards, while the quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in μg per 100 g of dry weight or in μg per 100 mL in the case of infusions and decoctions.

2.6. Analysis of phenolic compounds and antioxidant activity of hydromethanolic extracts and aqueous consumption forms

2.6.1. Preparation of the hydromethanolic extracts

The lyophilized plant material (1 g) was submitted to an extraction with a methanol:water mixture (80:20, *v/v*; 30 mL) at 25 °C and 150 rpm for 1 h, followed by filtration through a Whatman No. 4 filter paper. The residue was then extracted with one additional 30-mL portion of the hydromethanolic mixture. The combined extracts were evaporated under reduced pressure (R-210 rotary evaporator; Büchi, Flawil, Switzerland) and further lyophilized.

2.6.2. Phenolic compounds

The lyophilized extracts, infusions and decoctions were redissolved in methanol:water (80:20, *v/v*) and pure water, respectively, to determine the phenolic profiles by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA), as previously described (Guimarães et al., 2013). Detection was carried out with a diode array detector (DAD) using 280 nm and 370 nm as the preferred wavelengths connected in line with a mass spectrometer (API 3200 Qtrap; Applied Biosystems, Darmstadt, Germany). The phenolic compounds were identified by comparison of their retention times, UV–Vis and mass spectra with those obtained from standard compounds, if existing. Otherwise, peaks were tentatively identified by comparing the obtained information with previous studies performed in our laboratory (Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Dias, Barros, et al., 2016) and data in the literature (Del Bubba et al., 2012; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun, Liu, Yang, Slovin, & Chen, 2014; Yildirim & Turker, 2014). For quantitative analysis, individual standards calibration curves were constructed based on the area of the peaks recorded at 280 nm or 370 nm. For the identified phenolic compounds with no available commercial standard, the quantification was performed based on the calibration curve of a similar compound belonging to the same phenolic group. The results were expressed in mg per g of plant material.

2.6.3. Antioxidant activity

The lyophilized extracts, infusions and decoctions were redissolved in methanol:water (80:20, *v/v*) and water, respectively, to obtain stock solutions of 0.625 mg/mL, which were further diluted to obtain a range of six concentrations below the stock solution for antioxidant activity evaluation.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT), mixing 270 μL of DPPH solution (6×10^5 mol/L) with 30 μL of sample concentrations; mixtures were left for one hour in the dark at room temperature. The percentage of DPPH discoloration was calculated using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Barros, Heleno, Carvalho, & Ferreira, 2009; Barros et al., 2013).

Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+} by mixing an aliquot of the extract solutions (0.5 mL) with 0.5 mL of sodium phosphate buffer (200 mmol/L, pH 6.6) and 0.5 mL of potassium ferricyanide (1% *w/v*) and incubating at 50 °C for 20 min. The mixture (0.8 mL) was then placed in 48-well microplates containing 0.8 mL of deionised water and 0.16 mL of ferric chloride (0.1% *w/v*), measuring the absorbance at 690 nm in the microplate reader mentioned above (Barros et al., 2009; Barros et al., 2013). Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; briefly a 0.2 mg/mL solution of β-carotene was prepared to which was added linoleic acid (40 mg), Tween 80 (400 mg) and distilled water (100 mL) in a flask, followed by vigorous shaking. Different concentrations of the extracts (0.2 mL) were added to test tubes and mixed with aliquots of the previously prepared emulsion (4.8 mL) and zero time absorbance was measured at 470 nm. The test tubes were put in a water bath at 50 °C for 2 h, after which the absorbance was measured. The neutralization of linoleate free radicals prevents β-carotene bleaching, which is measured by the formula:

$$(\beta - \text{carotene absorbance after 2 h of assay/initial absorbance}) \times 100$$

(Barros et al., 2009; Barros et al., 2013).

Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS). Firstly, a pig brain tissue (*Sus crofa*) homogenate was prepared with ice-cold Tris-HCl buffer (20 mM, pH 7.4). The obtained supernatant was incubated with extract solutions (0.2 mL), $FeSO_4$ (10 μM; 0.1 ml) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was then stopped with 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. The colour intensity of the malondialdehyde-

thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the formula: $[(A - B)/A] \times 100\%$,where A and B were the absorbances of the control and the sample solution, respectively (Barros et al., 2013; Barros, Heleno, Carvalho, and Ferreira, 2009). The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

2.7. Statistical analysis

All the extractions were performed in triplicate and all the assays were also carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using Student's *t*-test, in order to determine the significant difference between two different samples, with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program.

3. Results and discussion

3.1. Nutritional and chemical characterization of the lyophilized plant material and aqueous consumption forms

Data on the nutritional and chemical composition of the *in vitro* cultured vegetative parts of *F. vesca* are shown in Table 1. Carbohydrates (including fiber) were the most abundant macronutrient (84 g/100 g dw), followed by proteins, ash and fat (7, 6.5 and 2 g/100 g dw, respectively). Compared to wild grown vegetative parts of *F. vesca* (Dias, Barros, Morales et al., 2015), the *in vitro* sample had higher content of protein and lower content of ash, whereas the values of fat and carbohydrates were similar.

Fourteen different fatty acids were found, more than half being polyunsaturated fatty acids (PUFA), mainly linoleic acid (C18:2*n*–6, 16%) and γ-linolenic acid (C18:3*n*–6, 38%). Palmitic acid (C16:0) was also found at high levels (22%). Dias, Barros, Morales et al. (2015) reported lower percentages of total polyunsaturated fatty acids (41%) and higher levels of saturated fatty acids (53%) in wild-grown vegetative parts of *F. vesca*. These results are motivating, since PUFA are components of membrane phospholipids, serve as precursors of some hormones with vital roles in the human body and are also important in the protection against some diseases, such as rheumatoid arthritis, psoriasis and some age-related diseases, such as Alzheimer's (Patil & Gislerød, 2006).

The profile of soluble sugars in the lyophilized plant material (Table 1) and in the aqueous preparations (Table 2) was very similar, glucose being the most abundant in all samples (4 g/100 g dw in the dry sample, 9 mg/100 mL in the infusion and 10 mg/mL in the decoction). Fructose was the second major sugar found in the lyophilized plant material and in the decoction (3 g/100 dw and 7 mg/100 mL, respectively), while for the infusion sucrose (6 mg/100 mL) appeared as the second major sugar. Compared with the results obtained by Dias, Barros, Morales, et al. (2015), the *in vitro* sample showed higher content of soluble sugars than the wild grown vegetative parts (total sugars = 6 g/100 g dw); furthermore, xylose was not previously described in the dry sample of *F. vesca*. These findings might indicate that the plant is producing larger amounts of sugars to maintain its vital functions of growth and development, since it is limited by the *in vitro* culture itself. *In vitro* plants have an incipient photosynthesis, and for that a higher amount of sugars in the medium is required, but some plants, under *in vitro* conditions, have a photosynthetic apparatus more developed than others, and if so, they can produce and store more sugars, like glucose, mannose, xylose or even raffinose; the type of sugar depends on the type of transportation in phloem, and that depends on the genetic characteristics of the plant species (Yaseen, Ahmad, Sablok, Standardi, & Hafiz, 2013).

Table 1
Nutritional value, fatty acids, soluble sugars, organic acids and tocopherols content of *in vitro* cultured vegetative parts from wild *Fragaria vesca* L. (mean ± SD).

Nutritional value	(g/100 g dw)	Soluble sugars	g/100 g dw
Fat	2.37 ± 0.01	Xylose	0.98 ± 0.02
Proteins	7.27 ± 0.12	Fructose	2.55 ± 0.17
Ash	6.53 ± 0.20	Glucose	3.94 ± 0.17
Total carbohydrates	83.83 ± 0.06	Sucrose	2.20 ± 0.01
Energy (kcal/100 g dw)	385.73 ± 0.57	Trehalose	0.35 ± 0.06
		Sum	10.04 ± 0.26
Fatty acids	(relative percentage)	Organic acids	g/100 g dw
C6:0	0.16 ± 0.01	Oxalic acid	3.76 ± 0.06
C8:0	0.34 ± 0.01	Quinic acid	0.85 ± 0.05
C10:0	0.22 ± 0.02	Shikimic acid	0.002 ± 0.001
C12:0	2.65 ± 0.12	Ascorbic acid	0.02 ± 0.01
C14:1	3.03 ± 0.07	Succinic acid	1.58 ± 0.20
C15:1	0.61 ± 0.04	Fumaric acid	tr
C16:0	21.37 ± 0.17	Sum	6.20 ± 0.21
C16:1	0.56 ± 0.09		
C17:0	0.57 ± 0.004		
C18:1n9	5.62 ± 0.08		
C18:2n6	16.11 ± 0.05		
C18:3n6	37.54 ± 0.46	Tocopherols	mg/100 g dw
C20:1	6.85 ± 0.05	α-Tocopherol	98.54 ± 0.90
C22:1n9	4.38 ± 0.01	β-Tocopherol	4.90 ± 0.04
SFA	**25.01 ± 0.15**	γ-Tocopherol	24.86 ± 0.23
MUFA	**21.34 ± 0.56**	δ-Tocopherol	11.04 ± 0.10
PUFA	**53.56 ± 0.41**	Sum	139.35 ± 1.27

nd- not detected; tr- traces. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. Calibration curves for organic acids: oxalic acid ($y = 9x106\ x + 377946$, $R^2 = 0.994$); quinic acid ($y = 6010607\ x + 46061$, $R^2 = 0.9995$); shikimic acid ($y = 7x107\ x + 175156$, $R^2 = 0.9999$); ascorbic acid ($y = 108\ x + 751815$, $R^2 = 0.998$); succinic acid ($y = 603298\ x + 4994.1$, $R^2 = 1$) and fumaric acid ($y = 154862\ x + 1x106$, $R^2 = 0.9977$). (<LOD: 12.6, 24, 6, 3, 19 and 0.080 μg/mL for oxalic, quinic, shikimic, ascorbic, succinic and fumaric acid respectively); (<LOQ: 42, 81, 19, 11, 64 and 0.26 μg/mL for oxalic, quinic, shikimic, ascorbic, succinic and fumaric acid respectively).

Table 2
Soluble sugars, organic acids and tocopherols contents in infusions and decoctions prepared from *in vitro* cultured vegetative parts of wild *Fragaria vesca* L. (mean ± SD).

Soluble sugars	Infusions mg/100 mL	Decoctions mg/100 mL	*t-Student p-value*
Xylose	2.85 ± 0.07	2.89 ± 0.20	0.572
Fructose	6.12 ± 0.15	7.15 ± 0.39	< 0.001
Glucose	9.49 ± 0.05	10.14 ± 0.80	0.013
Sucrose	6.48 ± 0.27	3.29 ± 0.20	< 0.001
Trehalose	1.17 ± 0.14	0.66 ± 0.14	< 0.001
Sum	26.13 ± 0.23	24.13 ± 1.46	< 0.001
Organic acids	mg/100 mL	mg/100 mL	
Oxalic acid	6.44 ± 0.01	5.55 ± 0.01	< 0.001
Quinic acid	4.958 ± 0.003	5.572 ± 0.001	< 0.001
Shikimic acid	0.086 ± 0.001	0.117 ± 0.001	< 0.001
Fumaric acid	tr	tr	–
Sum	11.48 ± 0.26	11.24 ± 0.24	< 0.001
Tocopherols	μg/100 mL	μg/100 mL	
α-Tocopherol	0.16 ± 0.02	0.17 ± 0.01	0.310
β-Tocopherol	1.82 ± 0.08	1.49 ± 0.01	< 0.001
Sum	1.98 ± 0.06	1.66 ± 0.01	< 0.001

tr- traces. Calibration curves for organic acids: oxalic acid ($y = 9 \times 106\ x + 377946$, $R^2 = 0.994$); quinic acid ($y = 6010607\ x + 46061$, $R^2 = 0.9995$); shikimic acid ($y = 7 \times 107\ x + 175156$, $R^2 = 0.9999$); ascorbic acid ($y = 108\ x + 751815$, $R^2 = 0.998$); succinic acid ($y = 603298\ x + 4994.1$, $R^2 = 1$) and fumaric acid ($y = 154862\ x + 1 \times 106$, $R^2 = 0.9977$).(<LOD: 12.6, 24, 6 and 0.080 μg/mL for oxalic, quinic, shikimic and fumaric acid respectively); (<LOQ: 42, 81, 19 and 0.26 μg/mL for oxalic, quinic, shikimic and fumaric acid respectively).

Regarding organic acids, oxalic acid was the major one found in the lyophilized plant material (3.76 g/100 g dw) followed by succinic acid (1.58 g/100 g dw); other acids, and among them ascorbic acid, were found at very low levels. Oxalic acid was also the predominant acid found in the infusions, although in that case followed by quinic acid (6 and 5 mg/100 mL, respectively), and quite similar amounts of these two organic acids were found in decoctions. As for sugars and fatty acids, the organic acids content in the *in vitro* cultured samples was significantly higher than the one reported by Dias, Barros, Morales, et al. (2015) in wild-grown vegetative parts and corresponding infusions (5.48 g/100 g dw, 9.99 mg/100 mL, respectively).

The four tocopherol isoforms were found in the lyophilized plant material, with α-tocopherol as predominant (99 mg/100 g dw) followed by γ-tocopherol (25 mg/100 g dw). However, only α- and β-tocopherol were detected in the infusions and decoctions, the latter being the major one in both preparations, as can be seen in Fig. 1. The lower content of tocopherols in the aqueous preparations was expected, due to their lipophilic character. Quite interestingly, the lyophilized plant material, infusions and decoctions of the *in vitro* cultured samples herein studied showed much higher tocopherol levels (139 mg/100 dw, 1.98 and 1.66 μg/100 mL, respectively) than the equivalent ones obtained from wild-grown vegetative parts of *F. vesca* (7 mg/100 g dw, 0.19 and 0.22 μg/100 mL, respectively), in which only one isoform (α-tocopherol) was reported in the infusions and decoctions (Dias, Barros, Morales, et al., 2015).

3.2. Phenolic profile and antioxidant activity of the hydromethanolic extracts and aqueous preparations

Table 3 presents the peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification of the phenolic compounds present in the hydromethanolic extracts, infusions and decoctions of the *in vitro* cultured vegetative parts of *F. vesca.* An example phenolic profile of the hydromethanolic extract recorded at 280 and 370 nm is shown in Fig. 2. Thirty different phenolic compounds where identified in the samples, four phenolic acids (peaks 8, 11, 12 and 14), twelve ellagic acid derivatives (peaks 1, 3, 9, 10, 15, 17, 18, 24, 25, 28, 29 and 30), four flavan-3-ols (peaks 2, 4, 6, and 7), nine flavonols (peaks 5, 13, 16, 19, 20, 21, 23, 26 and 27) and one dihydroflavonol (peak 22). The hydromethanolic extracts and the aqueous preparations showed a very similar profile, only distinguished at the quantification level and by the absence of some compounds in infusions and decoctions.

Most of the detected compounds (i.e., peaks 1–6, 8–10, 13, 15, 17–30) have been previously described in wild *F. vesca* and other *Fragaria* species (Del Bubba et al., 2012; Dias et al., 2016; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014), so that their identities are assumed herein. To the author's best knowledge, peaks 7, 11, 12, 14 and 16 have not been reported before in *F. vesca*. Although no information could be obtained regarding mass characteristics of peak 7, it was tentatively associated to a flavan-3-ol, based on the characteristic shape of its UV spectrum; the observed λ_{max} at 272 nm would point to a gallocatechin or a derived proanthocyanidin (e.g., a prodelphinidin), expected to have maximum wavelength at lower values than catechins and related procyanidins (278–280 nm). Peaks 11 and 12 were tentatively identified as coumaroylquinic acid isomers according to their pseudomolecular ion [M – H]– at *m*/*z* 337, releasing fragments at *m*/*z* 191 and *m*/*z* 163 corresponding to the deprotonated quinic acid and the coumaric acid moiety, respectively. Peak 14 was identified as feruloylquinic acid based on its pseudomolecular ion [M – H]– *m*/*z* at 367 and the production of a major daughter ion at *m*/*z* 193 [ferulic acid – H]–. Peak 16 showed a UV spectrum with λ_{max} at 368 nm, a pseudomolecular ion [M – H]– at *m*/*z* 477 and MS^2 fragments at *m*/*z* 315 and 301, which allowed its tentative identification as isorhamnetin-*O*-glucoside.

The methanolic extract presented higher concentrations of total phenolic compounds (44 mg/g) than the aqueous preparations (26–31 mg/g), mainly due to its greater content of ellagic acid derivatives (19 mg/g). Peak 17 (sanguiin H10 isomer) was the major compound found in the methanolic extracts, followed by

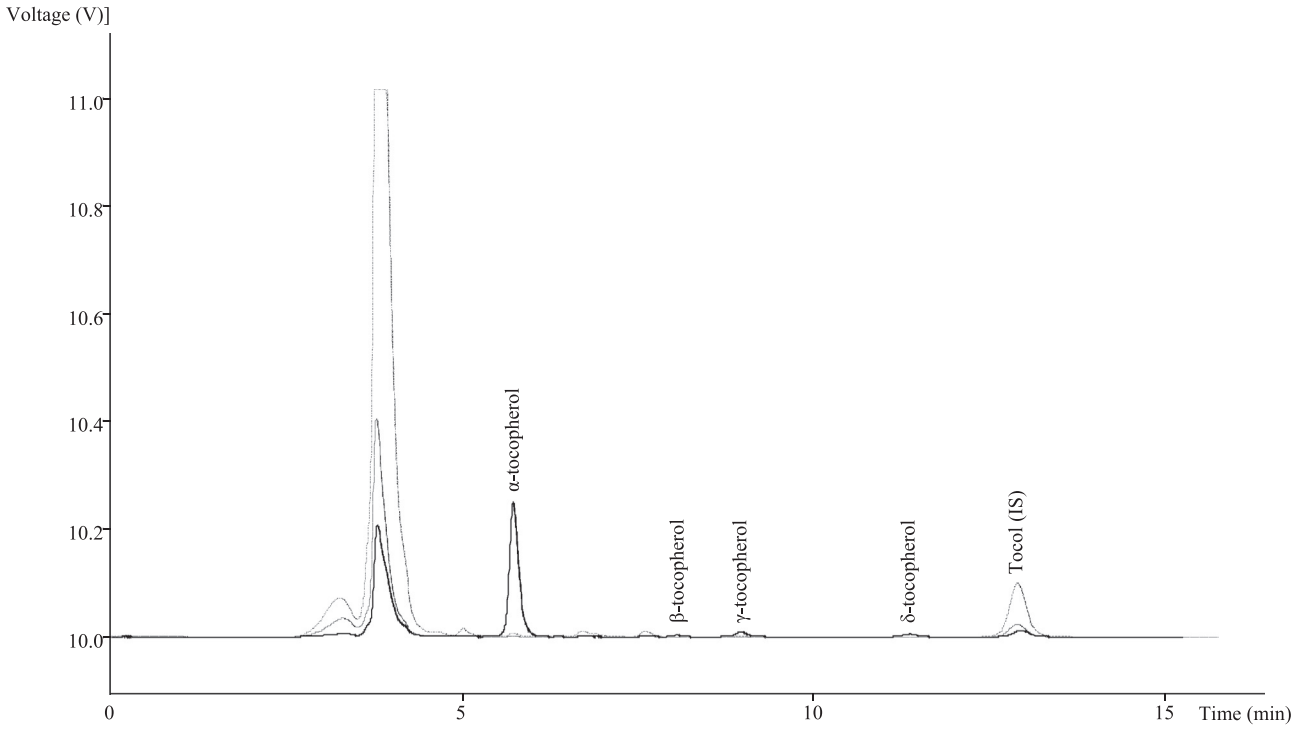

Fig. 1. Tocopherol profile of the hydromethanolic extract (——), infusion (- - - - -) and decoctions (-·-·-·-·) preparations of *in vitro* grown plants of *Fragaria vesca* L.

Table 3
Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification of phenolic compounds in hydromethanolic extracts, infusions and decoctions of the *in vitro* cultured vegetative parts of wild *Fragaria vesca* L.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative identification	Extracts	Infusions	Decoctions
						Phenolic compounds (mg/g dw)		
1	4.7	258	783	481(3),301(30)	Bis-HHDP-hexoside[B]	0.027 ± 0.001[a]	0.027 ± 0.001[c]	0.028 ± 0.003[b]
2	5.6	278	451	289(100)	(*Epi*)catechin hexoside[A]	0.060 ± 0.001[b]	0.081 ± 0.004[c]	0.173 ± 0.001[a]
3	6.05	256	783	481(25),301(14)	Bis-HHDP-hexoside[B]	0.006 ± 0.002[c]	0.015 ± 0.03[b]	0.033 ± 0.002[a]
4	6.87	278	577	451(33), 425(65), 407(100), 289(75), 287(17)	Procyanidin dimer[A]	0.09 ± 0.01[a]	0.071 ± 0.002[b]	0.07 ± 0.01[b]
5	7.19	356	639	463(69),301(59)	Quercetin glucuronyl-hexoside[E]	0.001 ± 0.0001[c]	0.003 ± 0.001[b]	0.004 ± 0.001[a]
6	7.76	278	289	245(35), 203(32), 137(32)	(+)-Catechin[A]	0.051 ± 0.002[c]	0.102 ± 0.001[b]	0.117 ± 0.004[a]
7	8.35	272	–	425(65), 407(), 289(100)	Gallocatechin-related flavan-3-ol[A]	0.039 ± 0.001[a]	0.033 ± 0.001[c]	0.051 ± 0.001[b]
8	9.63	332	355	193(18),175(100),161(20)	Ferulic acid hexoside[C]	0.006 ± 0.001[a]	0.005 ± 0.001[c]	0.006 ± 0.001[b]
9	14.5	270	935	633(25),301(21)	Galloyl-bis-HHDP-glucose isomer[B]	0.031 ± 0.001	nd	nd
10	15	372	463	301(100)	Ellagic acid hexoside[B]	0.006 ± 0.001[a]	0.007 ± 0.001[b]	0.005 ± 0.001[c]
11	15.45	316	337	191(7),173(35),163(10),155 (5)	Coumaroylquinic acid isomer[F]	0.008 ± 0.001a	0.010 ± 0.001[b]	0.010 ± 0.001[b]
12	15.7	316	337	191(8),173(38),163(12),155 (6)	Coumaroylquinic acid isomer[F]	0.003 ± 0.001[b]	0.009 ± 0.002[a]	0.005 ± 0.001[b]
13	16.61	352	623	301(100)	Quercetin rhamnosyl-glucuronide[E]	0.003 ± 0.001[b]	0.004 ± 0.001[b]	0.005 ± 0.001[a]
14	16.75	320	367	193(100),191(16),173 (14),149(25)	Feruloylquinic acid[C]	0.003 ± 0.001[c]	0.008 ± 0.001[b]	0.007 ± 0.001[a]
15	17.07	372	433	301(100)	Ellagic acid pentoside[B]	0.004 ± 0.001	tr	nd
16	17.59	368	477	315(35),301(100)	Isorhamnetin-O-glucoside[D]	0.016 ± 0.001[a]	0.021 ± 0.001[b]	0.017 ± 0.001[c]
17	17.93	262	1567	935(95), 783(5),631(2),613 (13), 301(6)	Sanguiin *h*10 isomer[B]	0.146 ± 0.002	nd	nd
18	19.29	250/sh370	447	301(100)	Ellagic acid rhamnoside[B]	0.004 ± 0.001[b]	0.011 ± 0.001[a]	0.006 ± 0.001[b]
19	19.3	346	607	285(100)	Kaempferol rhamnosyl-glucuronide[H]	0.01 ± 0.001[b]	0.015 ± 0.001[b]	0.019 ± 0.001[a]
20	19.87	356	477	301(100)	Quercetin glucuronide[E]	0.007 ± 0.001[a]	0.010 ± 0.001[c]	0.010 ± 0.001[b]
21	20.04	354	637	315(95),300(26)	Methylquercetin rhamnosyl glucuronide[E]	0.003 ± 0.001[c]	0.005 ± 0.001[b]	0.006 ± 0.001[a]
22	20.18	292/sh338	435	303(100)	Taxifolin-pentoside[G]	0.039 ± 0.001[a]	0.048 ± 0.002[b]	0.035 ± 0.004[c]
23	20.56	356	463	301(100)	Quercetin 3-O-glucoside[E]	0.003 ± 0.001	nd	nd
24	21.11	254/sh370	301	284(4),256(3),229(4), 185(4)	Ellagic acid[B]	0.012 ± 0.001[b]	0.037 ± 0.001[a]	tr
25	23.35	378	447	315(28),300(100)	Methyl ellagic acid pentoside[B]	tr	tr	0.034 ± 0.002
26	23.9	348	461	285(100)	Kaempferol-glucuronide[H]	0.004 ± 0.001[a]	0.006 ± 0.001[b]	0.006 ± 0.001[a]
27	24.21	348	447	285(100)	Kaempferol-hexoside[H]	0.002 ± 0.001[a]	0.002 ± 0.001[c]	0.002 ± 0.001[b]
28	24.83	364	447	315(12),300(100)	Methyl ellagic acid pentoside[B]	tr	tr	tr
29	25.26	248/sh372	461	315(89),301(38)	Dimethyl ellagic acid pentoside[B]	0.024 ± 0.001[a]	0.021 ± 0.001[b]	0.011 ± 0.001[c]
30	26.98	368	461	315(37),301(100)	Dymethyl ellagic acid pentoside[B]	tr	tr	tr
					Total phenolic acids	0.019 ± 0.001[b]	0.032 ± 0.001[a]	0.027 ± 0.001[c]
					Total ellagic acid derivatives	0.26 ± 0.01[a]	0.119 ± 0.001[b]	0.118 ± 0.001[b]
					Total flavan 3-ols	0.089 ± 0.001[a]	0.113 ± 0.002[b]	0.104 ± 0.006[c]
					Total flavonols	0.235 ± 0.003[b]	0.287 ± 0.002[c]	0.409 ± 0.003[a]
					Total phenolic compounds	0.6 ± 0.01[a]	0.551 ± 0.003[c]	0.658 ± 0.001[b]

Different letters mean significant statistical differences between samples ($p<0.05$), where "a" and "c" correspond to the highest and lowest values, respectively. tr-traces; nd- not detected. Standard calibration curves: (A) catechin ($y = 158.42\,x + 11.38$, $R^2 = 0.999$); (B) ellagic acid ($y = 32.748\,x + 77.8$, R^2=0.9994); (C) ferulic acid ($y = 525.36\,x + 233.82$, $R^2 = 0.9994$); (D) isorhametin-3-*O*-glucoside ($y = 218.26\,x - 0.98$, $R^2 = 1$); (E) quercetin-3-*O*-glucoside ($y = 253.52\,x - 11.615$, $R^2 = 0.9984$); (F) *p*-coumaric acid ($y = 706.09\,x + 1228.1$, $R^2 = 0.9989$); (G) taxifolin ($y = 224.31\,x + 148.41$, $R^2 = 0.999$); (H) kaempferol-3-*O*-glucoside ($y = 288.55\,x$ -4.0503, $R^2 = 1$).

peak 4 (procyanidin dimer). Different observations regarding the phenolic profile of *in vitro*-grown leaves of *F. vesca* were made by Yildirim and Turker (2014), who only reported two compounds in common with those detected in our study (i.e., (+)-catechin and a procyanidin dimer), and in much lower amounts.

Smaller contents of phenolic compounds were determined in the present study than previously found in wild grown vegetative parts (Dias, Barros, Fernandes, et al., 2015a). A possible explanation might be the short stationary phase in the growth of the *in vitro*-cultured plants, which would lead to lower yields in the production of secondary metabolites, due to the inhibition of the action of enzymes normally present in mature plants (Dias et al., 2016). Furthermore, *in vitro*-grown plants are not as subjected to environmental stress as wild plants, a factor that is known to influence phenolic accumulation. All in all, this could mean that *in vitro*-grown *F. vesca* would need to be elicited to produce higher amounts of phenolics.

3.3. Antioxidant activity of the hydromethanolic extracts and aqueous preparations

The results on the antioxidant activity of the hydromethanolic extract, infusions and decoctions of *in vitro* cultured vegetative parts are collected in Table 4. The hydromethanolic extract showed the highest DPPH-scavenging activity and reducing power (EC_{50} = 83 and 57 μg/mL, respectively), while for β-carotene bleaching inhibition and TBARS inhibition the lowest EC_{50} values were observed for the infusions (EC_{50} = 52 and 25 μg/mL, respectively). The results found for reducing power can be moderately correlated with the contents of phenolic acid derivatives in the samples ($r^2 = 0.777$), while for TBARS inhibition the results were highly correlated with these compounds ($r^2 = 0.903$), especially with ellagic acid derivatives ($r^2 = 0.9908$), as well as with flavonols ($r^2 = 0.915$).

The antioxidant activities found for the hydromethanolic extract in the DDPH scavenging, reducing power and β-carotene assays were higher than those observed for the extracts of wild grown vegetative parts of *F. vesca* L., despite the latter contained higher concentrations of phenolic compounds (138 mg/g) (Dias, Barros, Fernandes, et al., 2015). This could be due to the different phenolic profiles existing in both types of samples, but also to the presence of other components in the extracts, such as sugars, organic acids or tocopherols, which occur in higher levels in the *in vitro*-cultured sample, and may also have an influence on the antioxidant potential.

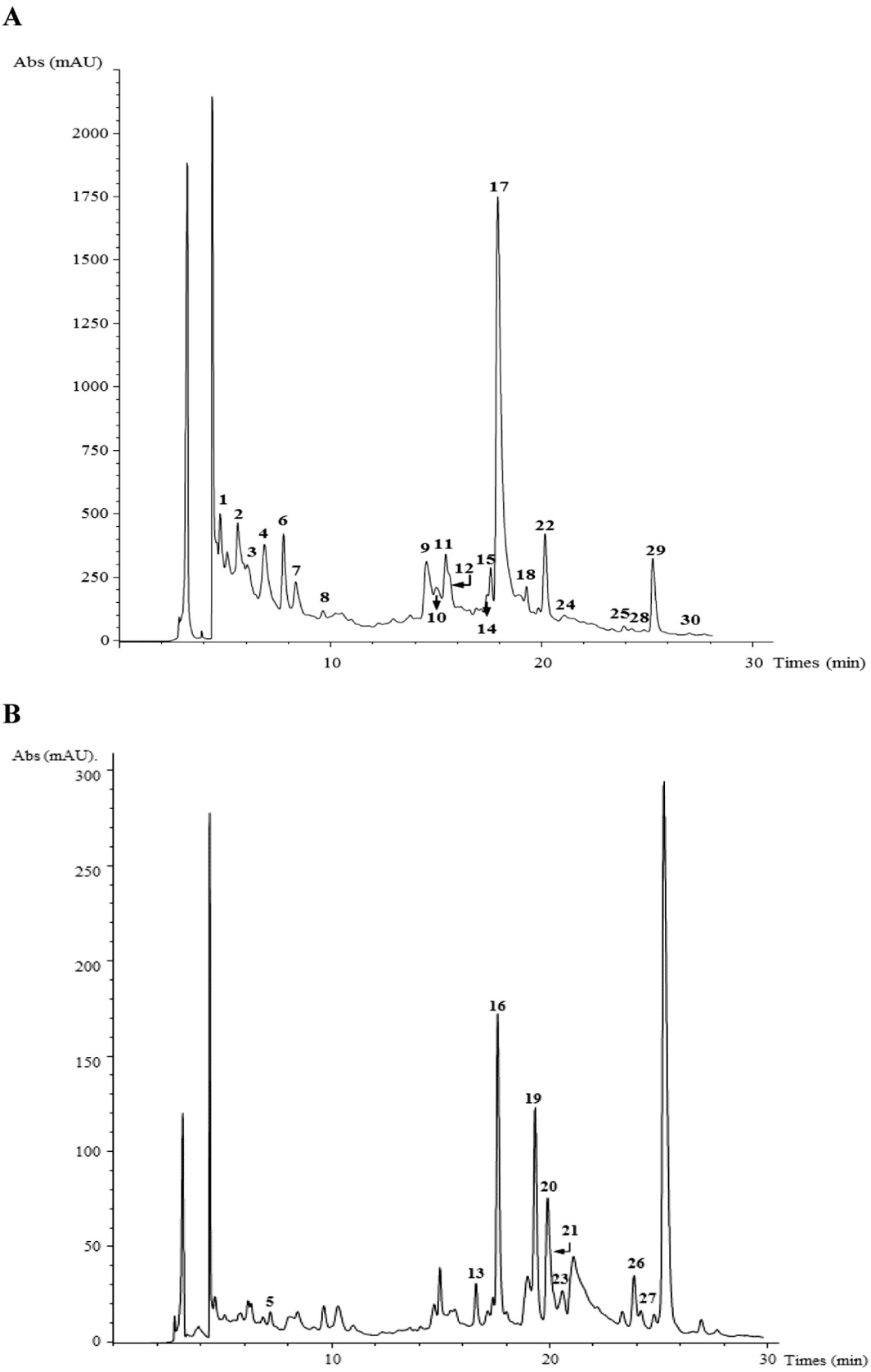

Fig. 2. HPLC chromatograms recorded at 280 nm (A) and 370 nm (B) showing the phenolic profile of the hydromethanolic extract of the *in vitro* cultured *Fragaria vesca* L.

Overall, the plant tissue culture technique applied to *Fragaria vesca* L. proved to be a suitable approach to obtain higher contents of proteins, polyunsaturated fatty acids, soluble sugars, organic acids (including ascorbic acid) and tocopherols (mainly α-tocopherol). Furthermore, the hydromethanolic extracts of the *in vitro*-grown samples showed greater antioxidant activity than

Table 4
Antioxidant activity of the hydromethanolic extracts, infusions and decoctions of *in vitro* cultured vegetative parts of wild *Fragaria vesca* L.

EC_{50} values (μg/mL)	Hydromethanolic	Infusion	Decoction
DPPH scavenging activity	82.5 ± 3.1[b]	86.9 ± 0.9[ab]	93.6 ± 10.1[a]
Reducing power	57.0 ± 0.1[c]	75.9 ± 0.4[a]	62.0 ± 0.3[b]
β-carotene bleaching inhibition	54.4 ± 1.9[a]	52.4 ± 1.0[b]	54.2 ± 0.1[a]
TBARS inhibition	230.3 ± 16.1[a]	25.3 ± 0.8[b]	27.1 ± 1.6[b]

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 43.03 ± 1.71 μg/mL (DDPH), 29.62 ± 3.15 μg/mL (reducing power), 2.63 ± 0.14 μg/mL (β-carotene bleaching inhibition) and 3.73 ± 1.9 μg/mL (TBARS inhibition). Different letters mean significant statistical differences between samples ($p<0.05$), where "a" and "c" correspond to the highest and lowest values, respectively.

the ones obtained from wild-grown *F. vesca*. In contrast, although the phenolic profile was similar to that observed in wild-grown plants, lower levels of total phenolic compounds were accumulated in the *in vitro*-cultured samples. Further studies are required to check if this limitation might be overcome by elicitation of plant growth.

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