Nutritional and *in vitro* antioxidant properties of edible wild greens in Iberian Peninsula traditional diet

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Abstract

Wild greens are nutritionally well-balanced vegetables. Herein, nutritional and *in vitro* antioxidant properties of the sprouts of three commonly used species were determined. Wild asparagus revealed the highest levels of moisture (84.6 g/100 g fw), ash (12.3 g/100 g dw), proteins (22.4 g/100 g dw), total sugars (9.24 g/100 g dw), including sucrose (4.27 g/100 g dw) and of the essential n-6 fatty acid linoleic acid (44.5 %); white bryony gave the highest contents of reducing sugars, including glucose (2.97 g/100 g dw), essential n-3 fatty acid α-linolenic acid (70.3 %), and the best ratios of PUFA/SFA and n-6/n-3 fatty acids (3.59 and 0.0907, respectively); black bryony showed the highest concentrations of carbohydrates (69.3 g/100 g dw), fructose and trehalose (3.83 and 1.34 g/100 g dw, respectively). Besides their culinary characteristics, their high content in vitamins (asparagus, 135 and 142 mg/100 g dw of total tocopherols and ascorbic acid respectively), chlorophylls (white bryony, 50.9 mg/100 g dw), carotenoids (23.3 mg/100 g dw) and phenolics (black bryony, 759 mg GAE/g extract), together with the antioxidant properties (EC_{50} values lower than 640 μg/ml) and potential health benefits increase their importance in traditional as well as in contemporary diets.

Keywords: Asparagus acutifolius; *Bryonia dioca*; *Tamus communis*; Wild edibles; Nutrients; Antioxidants.

1. Introduction

2. Materials and methods

2.1. Samples

Samples where gathered in 2009 early spring in Bragança, North-eastern Portugal, according local consumers' recommendations. The shoots of asparagus correspond to the young stems that sprout from the underground rhizome, more or less 20cm long. White bryony shoots are the soft climbing young stems with tendrils and the first 6-8 immature leaves (not yet fully expanded). Black bryony shoots match with the annual, climbing, lengthwise striated, young stems of about 20cm long, with primordia of leaves and spikes (immature floral buds) at the tip.

Morphological key characters from the Flora Iberica (Castroviejo, 2005) were used for white bryony identification and the Nova Flora de Portugal (Franco & Afonso, 1994) for asparagus and black bryony determination. Voucher specimens are deposited in the Escola Superior Agrária de Bragança herbarium (BRESA). The samples were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and kept in the best conditions for subsequent use.

2.2. Standards and Reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% 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). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Nutritional value

2.3.1. Macronutrients. The samples were analysed for chemical composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N × 6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. Reducing sugars were determined by DNS (dinitrosalicylic acid) method. Total energy was calculated according to the following equations: Energy (kcal) = 4 × (g protein +g carbohydrate) + 9 × (g lipid).

2.3.2. Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described by Barros, Carvalho, Morais, & Ferreira (2010). Dried sample powder (1.0 g) was spiked with the melezitose as internal standard (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 (Centorion K24OR- 2003 refrigerated centrifuge) at 15,000 g for 10 min. The supernatant was

concentrated at 60 ºC under reduced pressure 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. 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 RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 x 250 mm, 5 mm, 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 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 g per 100 g of dry weight.

2.3.3. Fatty Acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Barros et al., 2010), and after the following trans-esterification procedure: 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

106 was analyzed with a DANI model GC 1000 instrument equipped with a split/splitless
107 injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m x 0.32
108 mm ID x 0.25 µm d_f). The oven temperature program was as follows: the initial
109 temperature of the column was 50 ºC, held for 2 min, then a 10ºC/min ramp to 240 ºC
110 and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar),
111 measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. For each analysis 1
112 µl of the sample was injected in GC. Fatty acid identification was made by comparing
113 the relative retention times of FAME peaks from samples with standards. The results
114 were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in
115 relative percentage of each fatty acid.
116
117 *2.3.4. Tocopherols.* Tocopherols content was determined following a procedure
118 previously optimized and described by Barros, Heleno, Carvalho, & Ferreira (2010).
119 BHT solution in hexane (10 mg/ml; 100 µl) and IS solution in hexane (tocol; 50 µg/ml;
120 400 µl) were added to the sample prior to the extraction procedure. The samples (~500
121 mg) were homogenized with methanol (4 ml) by vortex mixing (1 min). Subsequently,
122 hexane (4 ml) was added and again vortex mixed for 1 min. After that, saturated NaCl
123 aqueous solution (2 ml) was added, the mixture was homogenized (1 min), centrifuged
124 (5 min, 4000g) and the clear upper layer was carefully transferred to a vial. The sample
125 was re-extracted twice with hexane. The combined extracts were taken to dryness under
126 a nitrogen stream, redissolved in 2 ml of n-hexane, dehydrated with anhydrous sodium
127 sulphate, filtered through 0.2 µm nylon filters from Whatman, transferred into a dark
128 injection vial and analysed by the HPLC system described above connected to a
129 fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and

emission at 330 nm. 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 mg per 100 g of dry sample.

2.3.5. Vitamin C. Vitamin C was determined according to the method of Klein and Perry (1982). A fine powder (20 mesh) of sample (150 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 within 30 min at 515 nm against a blank (Analytikijena 200-2004 spectrophotometer). Content of vitamin C was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml), and the results were expressed as mg per 100 g of dry weight.

2.3.6. Carotenoids and chlorophylls were determined according to the method of Nagata and Yamashita (1992). 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) = $- 0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$;

154 Chlorophyll a (mg/100 ml) = 0.999 × A_{663} - 0.0989 × A_{645}; Chlorophyll b (mg/100 ml)
155 = - 0.328 × A_{663} + 1.77 × A_{645}, and further expressed in mg per 100 g of dry weight.
156
157 *2.4. In vitro antioxidant properties*
158 *2.4.1. General.* Four in vitro assays were applied to evaluate the antioxidant activity of
159 the samples. Different concentrations of the extracts (7.8–500 µg/ml) were used to find
160 EC_{50} values. The extractions were performed using a fine dried powder (20 mesh; ~1g)
161 stirring with 50 ml of methanol at 25 ºC at 150 rpm for 1 h and filtered through
162 Whatman No. 4 paper. The residue was then extracted with one additional 50 ml portion
163 of methanol. The combined methanolic extracts were evaporated at 35ºC under reduced
164 pressure (rotary evaporator Büchi R-210), re-dissolved in methanol at 10 mg/ml, and
165 stored at 4 ºC for further use.
166
167 *2.4.2. DPPH radical-scavenging activity.* This methodology was performed using an
168 ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each
169 one of the 96-wells consisted of one of the different concentrations of the extracts (30
170 µl) and aqueous methanolic solution (80:20 v/v, 270 µl) containing DPPH radicals
171 ($6x10^{-5}$ mol/L). The mixture was left to stand for 60 min in the dark. The reduction of
172 the DPPH radical was determined by measuring the absorption at 515 nm. The radical
173 scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using
174 the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the
175 solution when the sample extract has been added at a particular level, and A_{DPPH} is the
176 absorbance of the DPPH solution (Barros et al., 2010). 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. This methodology was performed using the Microplate Reader described above. The different concentrations of the extracts (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 poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm (Barros et al., 2010). 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 (0.2 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 (Barros et al., 2010). 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 (0.2 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 (Barros et al., 2010). 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.

2.4.6. Phenolics and flavonoids

Phenolics were estimated based on procedures described by Wolfe, Wu, & Liu (2003)

225 with some modifications. An aliquot of the extract solution (1 ml) was mixed with
226 *Folin-Ciocalteu* reagent (5 ml, previously diluted with water 1:10 v/v) and sodium
227 carbonate (75 g/l, 4 ml). The tubes were vortexed for 15 s and allowed to stand for
228 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm.
229 Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the results were
230 expressed as mg of gallic acid equivalents (GAE) per g of extract.
231 Flavonoids were determined using the method of Jia Tang, & Wu (1999), with some
232 modifications. An aliquot (0.5 ml) of the extract solution was mixed with distilled water
233 (2 ml) and subsequently with $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$
234 solution (10%, 0.15 ml) was added and allowed to stand further 6 min, thereafter, NaOH
235 solution (4%, 2 ml) was added to the mixture. Immediately, distilled water was added to
236 bring the final volume to 5 ml. Then the mixture was properly mixed and allowed to
237 stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin
238 was used to calculate the standard curve (0.0156-1.0 mM) and the results were
239 expressed as mg of (+)-chatequin equivalents (CE) per g of extract.
240
241 *2.5. Statistical analysis*
242 For each one of the samples the assays were carried out in triplicate. The results are
243 expressed as mean values and standard deviation (SD). The results were analyzed using
244 one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$.
245 This treatment was carried out using SPSS v. 16.0 program.
246
247 **3. Results and discussion**
248 *3.1. Nutritional value*

249 The results of the nutrients composition and energetic value (expressed on dry weight
250 basis) obtained for the studied wild edible greens are shown in **Table 1**. Asparagus
251 revealed the highest moisture content (84.6 g/100 g), while white bryony showed the
252 lowest contents (82.9 g/100 g). Carbohydrates, calculated by difference, were the most
253 abundant macronutrients and were higher for black bryony (69.3 g/100 g). This result
254 can be explained by the fact that black bryony' shoots are in general more fibrous than
255 the others. Protein (22.4 g/100 g) and ash (12.3 g/100 g) contents were higher in
256 asparagus. The asparagus'shoots include numerous small fleshy scale-shaped leaves and
257 are modified stems for storage, a type of morphology that may be responsible for a
258 higher value of protein content. The value of protein found in this sample was higher
259 than the concentration found in sea-asparagus, *Salicornia bigelovii* Torr., (13.3 g/100 g
260 of dry weight; Lu et al., 2010) but similar to the one found in cultivated asparagus
261 (*Asparagus officialis* L.) harvested in different months (Spring- 28.7 g/100 g; Summer-
262 28.4 g/100 g; Autumn- 29.1 g/100 g) (Shou, Lu, & Huang, 2007). Other authors (Turan,
263 Kordali, Zengin, Dursum, & Sezen, 2003) reported that minerals and protein contents of
264 twenty five wild vegetables were all higher than those of cultivated species, such as
265 spinach, pepper, lettuce, and cabbage. Fat predominated in white bryony (15.1 g/100 g),
266 contributing to its higher energy value (440 g/100 g). The studied wild greens showed
267 high nutritional value with a low energetic contribution of 397 kcal/100 g, on average.
268 The three wild edible greens presented fructose, glucose, sucrose and trehalose as main
269 sugars (**Table 1**). Sucrose predominates in asparagus while glucose was the most
270 abundant sugar in white and black bryony. Asparagus revealed the highest total sugars
271 content, with the highest levels of sucrose (non-reducing sugar) which is in agreement
272 with its lowest content in reducing sugars. The value of total sugars found in this sample

was lower than the concentration found in asparagus obtained by mother fern cultivation, and harvested in different months (Spring- 23.6 g/100 g; Summer- 17.0 g/100 g; Autumn- 20.2 g/100 g) (Shou et al., 2007). For those samples, in whole harvesting season, content of fructose was higher than of glucose and sucrose. However, under normal culture, asparagus gave higher sucrose content than that of glucose and fructose, similarly to the results obtained in the present study. Sugars are only a small part of carbohydrates (**Table 1**) that also include polysaccharides such as amide and cellulose.

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the ratios of PUFA/SFA and n-6/n-3 of the studied wild edible greens are shown in **Table 2**.The major fatty acid found in asparagus and black bryony was linoleic acid (C18:2n6; ~43%), while of α-linolenic acid (C18:3n3) predominated in white bryony (~70%), contributing to the prevalence of PUFA in all the samples (>68%). Fatty acids from n-6 series are biogenetic precursors of some physiologically important thromboxanes, leukotrienes and prostaglandins, hormones which are related to the inflammatory response. Moreover, the nutritional value of n-3 and n-6 fatty acids is widely known for its health beneficial effects (Guil, Torija, Giménez, & Rodriguez, 1996). Palmitic acid was the SFA most abundant in the samples (~16%). Twenty one fatty acids were identified and quantified.

The ratios of PUFA/SFA which should be above 0.45 for "good nutritional quality", and the n-6/n-3 fatty acids, which should be lower than 4.0, have been proposed as a means of measuring the quality of the fatty acids present in food (Guil et al., 1996). All the

297 wild greens showed PUFA/SFA ratios higher than 0.45 and n-6/n-3 ratios lower than
298 4.0 (**Table 2**); the best values were revealed by white bryony (3.59 and 0.0907,
299 respectively). According to the results obtained, the studied wild greens consumption
300 should be considered healthy.

301 Vitamins, carotenoids and chlorophylls contents were determined and the results are
302 given in **Table 3**. The four vitamers of tocopherols were detected, being α-tocopherol
303 the major compound in all the species. Asparagus presented the highest content of
304 tocopherols (135 mg/ 100 g of dry weight) with the highest levels of all the isoforms.
305 Vitamin C is an important nutrient in vegetables, but it was only detected in asparagus
306 (**Table 3**). Generally, fruits and vegetables show a gradual decrease in ascorbic acid
307 content as storage temperature and/or duration increases. The loss of ascorbic acid
308 content is most probably dominated by the presence of catalysts and oxidase enzymes,
309 such as polyphenol oxidase (PPO) to catalyse the oxidation especially at high
310 temperature (Lu et al., 2010). Asparagus sample revealed a concentration of vitamin C
311 (142 mg/100 g) higher than sea-asparagus (50.4 mg/100 g; Lu et al., 2010) and
312 cultivated asparagus from China (49.0 mg/100 g; Shou et al., 2007), but slightly lower
313 than cultivated asparagus from USA (~200 mg/100 g; Nindo et al., 2003). The studied
314 wild greens, particularly asparagus, contain high amounts of vitamins E and C. These
315 and other antioxidant micronutrients present in fresh fruits and vegetables might have
316 potential health-promoting effects (Flyman & Afolayan, 2006). Furthermore, vitamin C
317 can also act as a synergist with tocopherols by regenerating or restoring their
318 antioxidant properties (Nindo et al., 2003).

319 β-carotene, chlorophylls a and b were found in all the studied greens being the first
320 pigment higher in black bryony (23.3 mg/100 g dry weight; **Table 3**), while the others

predominated in white bryony (50.9 mg/100 g). Lycopene was not detected. Despite, some authors had reported a prevalence of carotene in wild relative to locally cultivated species (Flyman & Afolayan, 2006), the sample of wild asparagus herein studied gave lowest levels of β-carotene and chlorophylls than cultivated asparagus (24.0 and 64.7 mg/100g, respectively; Shou et al., 2007). This difference could be also due to the extraction methodology applied: acetone:hexane was used as solvent extraction in the present study, while the other authors used aqueous acetone; furthermore, we did not used sodium ascorbate to avoid pheophytin formation. Lu et al. (2010) reported even higher values for the mentioned pigments in sea-asparagus (~137 mg/100g of β-carotene and ~491 mg/100g of chlorophylls).

3.2. In vitro antioxidant properties

Fruits, vegetables and beverages contain a significant amount of flavonoids (flavonols, flavones, flavanones, flavans and anthocyanins). While there is no direct evidence that these antioxidants are central to the benefits of the Mediterranean Diet, indirect evidence from epidemiological data and the increasing understanding of their mechanisms of action suggest that antioxidants may play a major role (Trichopoulou et al., 2000). In the present study, phenolics were the major antioxidant components (258-759 mg GAE/g of extract; **Table 4**); black bryony revealed the highest content in phenolics and flavonoids. This sample had much higher contents than black bryony root methanolic extracts (Boumerfeg et al., 2009), that revealed only 312 mg phenolics/g and 119 flavonoids/g; it was possible to express the results by extract weigh because the authors published the extraction wield. Other authors also reported the phenolic contents in cultivated asparagus from China (Shou et al., 2007) and USA (Sun et al., 2007b), but

345 the results were expressed in a basis of fresh weigh or dry weight, making impossible
346 the comparison with the sample of wild asparagus herein studied, in which the results
347 are expressed by extract weight to a better correlation to the antioxidant properties.
348 Asparagus was ranked fourth in terms of total phenols content and first in terms of
349 antioxidant activity among 23 vegetables commonly consumed in the United States
350 (Vinson, Hao, Su, & Zubik, 1998).
351 The ranking of the antioxidant activity of the samples may vary with the analysis
352 methods. It is common to evaluate the antioxidant activity of plants using several
353 methods to measure various oxidation products (Sun et al., 2007b). Therefore, to
354 evaluate the antioxidant activity of the wild edible greens, four *in vitro* chemical and
355 biochemical assays using animal cells were performed: scavenging effects on DPPH
356 radicals- measuring the decrease in DPPH radical absorption after exposure to radical
357 scavengers, reducing power- measuring the conversion of a Fe^{3+}/ferricyanide complex
358 to Fe^{2+}, inhibition of β-carotene bleaching- measuring the capacity to neutralize the
359 linoleate-free radical and other free radicals formed in the system which attack the
360 highly unsaturated β-carotene models, and inhibition of lipid peroxidation in brain cells
361 homogenates- measuring the colour intensity of MDA-TBA complex.
362 All the samples showed antioxidant activity (**Table 4**) in the order of black bryony >
363 asparagus > white bryony (EC_{50} values lower than 0.5 mg/ml). Significantly negative
364 linear correlations (**Figure 1**) were established between the phenolics and flavonoids
365 content, and EC_{50} values of DPPH scavenging activity (determination coefficient 0.886
366 for phenolics and 0.909 for flavonoids; ***p<0.001), reducing power (determination
367 coefficient 0.568 for phenolics and 0.928 for flavonoids; ***p<0.001), β-carotene
368 bleaching inhibition (determination coefficient 0.590 for phenolics and 0.750 for

369 flavonoids, *** $p<0.001$) and TBARS inhibition (determination coefficient 0.874 for
370 phenolics and 0.597 for flavonoids, *** $p<0.001$). This proves that the wild green with
371 the highest bioactive compounds content is the most efficient in antioxidant activity
372 (with the lowest EC_{50} values). The correlations were slightly more significant for
373 flavonoids than for phenolics, and the highest determination coefficients were obtained
374 for DPPH and β-carotene bleaching inhibition assays. Sun et al. (2007b) also related a
375 correlation between the antioxidant activity of cultivated asparagus (measured by DPPH
376 and β-carotene bleaching assays) and the total flavonoid content. Furthermore, the same
377 others in other report (Sun et al., 2007a) described a significant correlation between
378 rutin content and antioxidant activity of processing asparagus, suggesting that this
379 flavonoid plays a key role in the antioxidant activity of *Asparagus officinalis*.
380 Black bryony, wild asparagus and white bryony proved to be vegetables with high
381 antioxidant activity. Antioxidants can scavenge free radicals and assist in the protection
382 of the human body from oxidative stress, related to several diseases including cancers
383 and heart diseases. Furthermore, they can be used as additives in the food industry
384 providing good protection against oxidative damage.
385
386 Overall, the studied wild greens were central to traditional diet in the Iberian peninsula
387 and, particularly, in Portugal and they have generally been overlooked as minor or
388 trivial constituents. They are nutritionally well-balanced vegetables; particularly
389 asparagus revealed the highest levels of moisture, ash, proteins, total sugars, including
390 sucrose and of the essential n-6 fatty acid linoleic acid; white bryony gave the highest
391 contents of reducing sugars, including glucose, essential n-3 fatty acid α-linolenic acid,
392 and the best ratios of PUFA/SFA and n-6/n-3 fatty acids; black bryony showed the

highest concentrations of carbohydrates, fructose and trehalose. Furthermore, they are a good source of important phytochemicals such as asparagus for vitamins, white bryony for chlorophylls and black bryony for β-carotene. Besides their culinary characteristics (smoothness, slipperiness, mouthfeel), their high content in antioxidants such as vitamins, carotenoids and phenolics, requires reconsideration of the role of these agents in the traditional as well as the contemporary diet. To increase their consumption it is important to take advantage of the health benefits of wild greens by investigating their nutritional value and *in vitro* antioxidant properties (like it was performed in the present work), but also their possible toxicity.

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Table 1. Moisture (g/100 g of fresh weight), nutrients (g/100 g of dry weight) and energetic value (Kcal/100 g of dry weight) of the edible wild greens (mean ± SD; n=3). In each row, different letters mean significant differences (p<0.05).

	Asparagus	White bryony	Black bryony
Moisture	84.6 ± 3.8 a	82.9 ± 2.3 c	83.3 ± 1.3 b
Ash	12.3 ± 0.0 a	8.79 ± 0.01 b	8.62 ± 0.15 c
Proteins	22.4 ± 0.1 a	16.6 ± 0.4 c	19.1 ± 0.8 b
Fat	3.99 ± 0.33 b	15.1 ± 1.9 a	3.05 ± 0.12 b
Carbohydrates	61.3 ± 0.3 b	59.5 ± 1.2 c	69.3 ± 0.7 a
Reducing sugars[a]	4.24 ± 0.08 c	6.62 ± 0.05 a	5.79 ± 0.13 b
Energy	371 ± 1 c	440 ± 7 a	381 ± 1 b
Fructose	2.49 ± 0.13 c	3.45 ± 0.08 b	3.83 ± 0.13 a
Glucose	1.98 ± 0.04 b	2.97 ± 0.09 a	1.80 ± 0.14 b
Sucrose	4.27 ± 0.12 a	0.572 ± 0.014 b	0.695 ± 0.05 b
Trehalose	0.497 ± 0.009 b	0.307 ± 0.005 c	1.34 ± 0.09 a
Total Sugars[b]	9.24 ± 0.28 a	7.30 ± 0.19 b	7.66 ± 0.13 b

[a]Reducing sugars were determined by DNS spectrophotometer assay; [b]Total sugars were determined by HPLC-RI.

514 **Table 2.** Composition of the edible wild greens in fatty acids (mean ± SD; n=3). In each
515 column different letters mean significant differences (p<0.05).
516

	Asparagus	White bryony	Black bryony
C6:0	0.0360 ± 0.0141	0.0550 ± 0.0007	0.0130 ± 0.0028
C8:0	0.0210 ± 0.0057	0.0160 ± 0.008	0.0195 ± 0.0064
C10:0	0.0585 ± 0.0035	0.0125 ± 0.0064	0.0265 ± 0.0007
C12:0	0.315 ± 0.008	0.0555 ± 0.0021	0.119 ± 0.002
C14:0	0.279 ± 0.011	0.238 ± 0.029	0.146 ± 0.006
C14:1	0.0300 ± 0.0042	0.0750 ± 0.0017	0.0270 ± 0.0014
C15:0	0.280 ± 0.021	0.0730 ± 0.0028	0.147 ± 0.005
C16:0	17.5 ± 0.2	13.5 ± 0.3	17.0 ± 0.7
C16:1	0.0925 ± 0.0049	0.0315 ± 0.0021	0.200 ± 0.004
C17:0	0.260 ± 0.001	0.230 ± 0.008	0.109 ± 0.006
C18:0	1.56 ± 0.19	2.27 ± 0.01	0.965 ± 0.049
C18:1n9c	4.94 ± 0.35	1.52 ± 0.09	7.51 ± 0.18
C18:2n6c	44.5 ± 1.3	6.39 ± 0.16	42.0 ± 0.3
C18:3n3	23.7 ± 0.9	70.3 ± 0.1	27.5 ± 0.4
C20:0	0.429 ± 0.066	0.307 ± 0.007	0.241 ± 0.035
C20:1c	0.262 ± 0.006	0.102 ± 0.001	0.406 ± 0.021
C20:2c	0.196 ± 0.017	0.0390 ± 0.0042	0.232 ± 0.023
C20:3n3+C21:0	0.104 ± 0.011	0.141 ± 0.008	0.0880 ± 0.0110
C22:0	2.22 ± 0.19	0.650 ± 0.019	0.748 ± 0.041
C23:0	0.610 ± 0.022	2.62 ± 0.21	0.575 ± 0.051
C24:0	2.62 ± 0.19	1.47 ± 0.16	1.92 ± 0.03
Total SFA	26.2 ± 0.1 a	21.4 ± 0.2 c	22.0 ± 0.3 b
Total MUFA	5.32 ± 0.35 b	1.72 ± 0.07 c	8.15 ± 0.21 a
Total PUFA	68.5 ± 0.4 c	76.8 ± 0.2 a	69.9 ± 0.1 b
PUFA/SFA	2.62 ± 0.02 c	3.59 ± 0.04 a	3.18 ± 0.05 b
n-6/n-3	1.87 ± 0.12 a	0.0907 ± 0.0021 c	1.52 ± 0.03 b

517 Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0);
518 Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic
519 acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid
520 (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3);
521 Arachidic acid (C20:0); Eicosenoic acid (C20:1c); *cis*-11,14-Eicosadienoic acid
522 (C20:2c); *cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0);
523 Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).

524 **Table 3.** Composition of the edible wild greens in vitamins, carotenoids and
525 chlorophylls (mg/100 g dry weight) (mean ± SD; n=3). In each row different letters
526 mean significant differences (p<0.05).

527

	Asparagus	White bryony	Black bryony
α-tocopherol	95.6 ± 4.7 a	37.4 ± 5.6 b	30.4 ± 0.7 b
β-tocopherol	0.739 ± 0.017 a	0.820 ± 0.017 a	0.707 ± 0.019 a
γ-tocopherol	37.7 ± 2.6 a	18.6 ± 0.2 b	19.5 ± 0.4 b
δ-tocopherol	1.14 ± 0.18 a	0.782 ± 0.045 b	1.41 ± 0.01 a
Total tocopherols	135 ± 8 a	57.6 ± 6.0 b	52.0 ± 1.0 b
Vitamin C	142 ± 12	nd	nd
β-carotene	12.1 ± 0.0 c	22.7 ± 0.2 b	23.3 ± 0.0 a
Lycopene	nd	nd	nd
Chlorophyll a	6.72 ± 0.00 c	39.4 ± 0.2 a	12.8 ± 0.0 b
Chlorophyll b	3.53 ± 0.01 c	11.5 ± 0.1 a	3.85 ± 0.01 b

528

529 nd- not detected.

Table 4. Extraction yields, composition in phenolics and flavonoids, and antioxidant activity (EC_{50} values, μg/ml) of the edible wild greens (mean ± SD; n=3). In each row different letters mean significant differences ($p<0.05$).

	Asparagus	White bryony	Black bryony
η (%)	29.7 ± 1.6	48.2 ± 3.3	23.4 ± 2.5
Phenolics (mg GAE/g extract)	624 ± 28 b	258 ± 22 c	759 ± 29 a
Flavonoids (mg CE/g extract)	57.8 ± 2.4 b	18.1 ± 1.2 c	150 ± 12 a
DPPH scavenging activity	423 ± 24 b	640 ± 49 a	203 ± 30 c
Reducing power	191 ± 12 b	204 ± 9 a	68.1 ± 3.6 c
β-carotene bleaching inhibition	166 ± 7 b	371 ± 5 a	70.7 ± 5.3 c
TBARS inhibition	105 ± 4 b	197 ± 10 a	94.7 ± 6.4 b

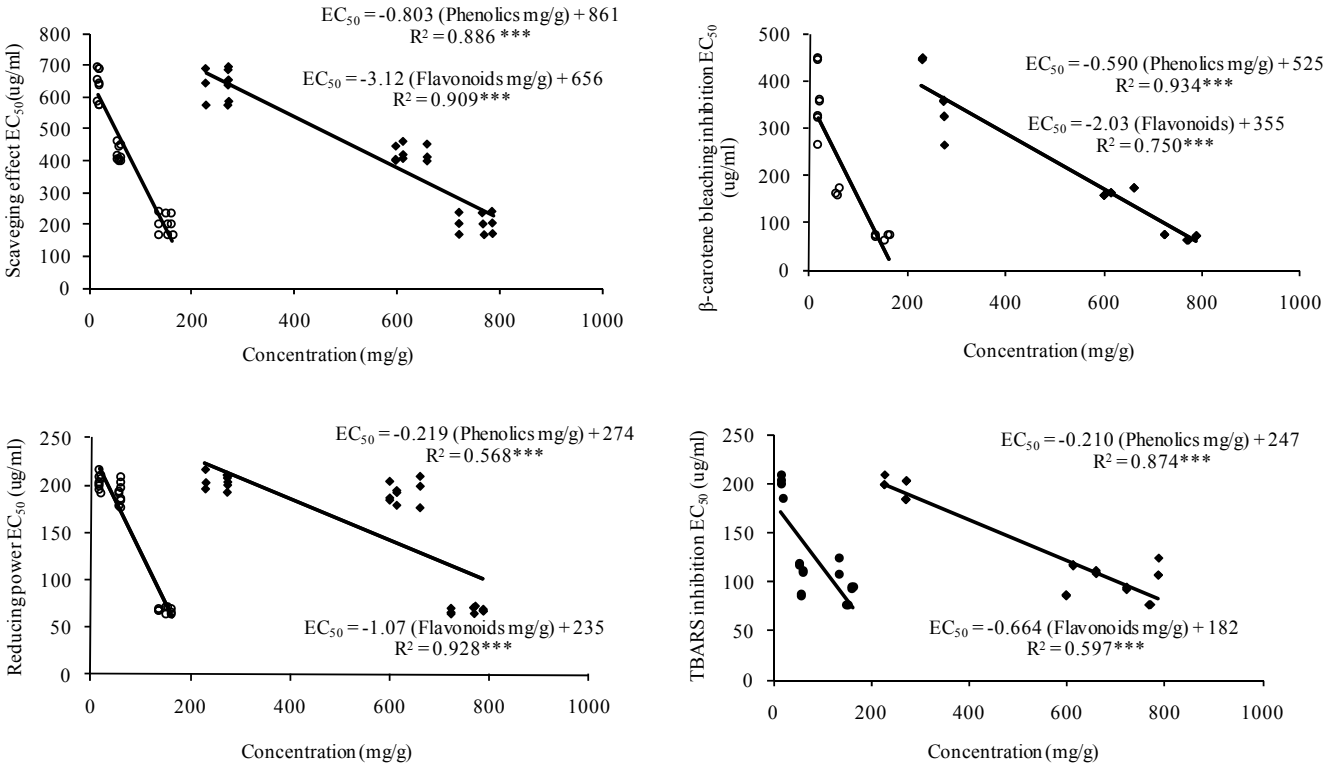

Figure 1. Correlation established between total phenolics (♦) and flavonoids (○) contents, and scavenging effect on DPPH radicals, reducing power, β-carotene bleaching inhibition and lipid peroxidation inhibition.