In vitro Antioxidant Properties and Characterization in Nutrients and Phytochemicals of Six Medicinal Plants from the Portuguese Folk Medicine

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ABSTRACT

Traditional ethnomedical use of plants is recognized as an important potential source of compounds used in mainstream medicine. Herein, the i*n vitro* antioxidant properties, nutrients and phytochemical composition of six medicinal plants widely used in the north-eastern Portuguese region were evaluated. The antioxidant activity was screened through: radical scavenging effects, reducing power, and inhibition of lipid peroxidation in brain homogenates. Nutrients and phytochemical characterization included determination of sugars by HPLC-RI, fatty acids by GC-FID, tocopherols by HPLC-fluorescence, phenolics, flavonoids, carotenoids and ascorbic acid, by spectrophotometric techniques. *Rubus ulmifolius* flowers revealed the highest antioxidant activity, and the highest contents in phenolics, flavonoids, ascorbic acid and lycopene. *Castanea sativa* flowers revealed the highest concentration of individual and total tocopherols, and total sugars, including the reducing sugars glucose and fructose. *Helichrysum stoechas* aerial parts gave the highest levels of β-carotene, *Matricaria recutita* aerial parts revealed the highest PUFA levels, including linoleic acid. The studied medicinal plants have interesting antioxidant properties and a phytochemical composition that could provide scientific evidence for some folk uses in the treatment of diseases related to the production of ROS and oxidative stress, but further experiments are required to explore the mechanisms of action.

***Keywords*:** Medicinal plants; Antioxidant properties; Phytochemicals; Portuguese ethnobotany

1. Introduction

More and more chronic diseases are crippling the ageing population: cancers, arthritis and arthrosis, cardiovascular and neurodegenerative diseases bring more people to hospitals and retirement boarding houses (Montagnier, 2009). At the individual level, there is a common biochemical denominator resulting from the summation of genetic, behavioural and environmental factors: oxidative stress. There are intrinsic factors for the generation of ROS (reactive oxygen species): dysfunction of mitochondria, thymic involution favouring chronic inflammation and infections (Montagnier, 2009).

Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in drug discovery and development (Mishra *et al.*, 2008). Particularly, the plant kingdom offers a wide range of natural antioxidants. However, little is known about the practical usefulness of most of them. Many herbal and plant infusions frequently used in folk medicine have antioxidative and pharmacological properties connected with the presence of phenolic compounds, especially flavonoids (Dawidowicz *et al.*, 2006). The biological, pharmacological and medicinal properties of this group of compounds have been extensively reviewed (Marchand, 2002).

In rural areas, such as the north-eastern region of Portugal, folk medicine and traditional healing practices often coexists with formalized and institutionalized medicine systems. Since a long time ago, several species from the local flora have become very popular and are widely consumed due to their pharmacological properties and therapeutic effects. These species are mainly recommended for disorders of the respiratory system, digestive system, inflammation, cholesterol and hypertension (Neves et al., 2009; Carvalho, 2010).

62 Ethnobotanical surveys conducted in this Portuguese region highlighted (Frazão-
63 Moreira et al., 2007; Carvalho, 2010; Carvalho and Morales, 2010) some widespread
64 examples of traditional plant use such as the decoction of upright catkins of the
65 Fagaceae *Castanea sativa* Miller for cold and caught, diarrhea and cholesterol; infusions
66 and decoctions of the flowering aerial parts of the Asteraceae *Centaurea paniculata* L.
67 for inflammation, *Helichrysum stoechas* (L.) Moench for the respiratory system and to
68 reduce fever, *Matricaria recutita* L. for cold, bronchitis, inflammation and as digestive;
69 decoctions of the inflorescences of the Fabaceae *Trifolium angustifolium* L. and of
70 flower buds and flowers of the Rosaceae *Rubus ulmifollius* Schott for stomachache and
71 diarrhea.
72 Although antioxidant properties of some of these plants, such as *Helichrysum stoechas*
73 (Carini *et al.*, 2001; Albayrak *et al.*, 2010), *Matricaria recutita* (Miliauskas *et al.*, 2004;
74 Schempp *et al.*, 2006) and *Rubus ulmifolius* (Dall'Acqua *et al.*, 2008; Martini *et al.*,
75 2009) have been investigated in different countries, there are no reports on material
76 collected in Portugal. *Castanea sativa* flowers antioxidant properties were reported by
77 our research group (Barreira *et al.*, 2008), but without a complete characterization of the
78 chemical constituents.
79 This work aims to study the antioxidant potential of six medicinal plants traditionally
80 used in the northeastern region of Portugal (*Castanea sativa* flowers, *Centaurea*
81 *paniculata, Helichrysum stoechas Matricaria recutita* and *Trifolium angustifolium*
82 flowering aerial parts, and *Rubus ulmifolius* flower buds and flowers), characterize their
83 nutrients and phytochemical composition, and find correlations with their folk
84 medicinal uses.
85

2. Materials and methods

2.1. Samples

All the samples were collected in 2009, in the Natural Park of Montesinho territory, Trás-os-Montes, North-eastern Portugal, considering the Portuguese folk pharmacopoeia, the local medicinal criteria of use and the plants growth patterns. From *Castanea sativa,* the upright catkins during anthesis in late summer; from *Centaurea paniculata, Helichrysum stoechas Matricaria recutita* and *Trifolium angustifolium,* the inflorescences and leafy flowering stems of about 15 cm long, in late spring and early summer; the flower buds and fully opened flowers (each part constituting one different sample according to folk uses) of *Rubus ulmifollius* in spring.

Morphological key characters from the Flora Iberica (Castroviejo 1991, 2001 and 2007) and the "Nova Flora de Portugal" (Franco 1994) were used for plant identification. Voucher specimens are deposited in the "Herbarium of Escola Superior Agrária de Bragança" (BRESA). Each sample was 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 (Lab-Scan, Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO), as also other individual fatty acid isomers, 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 (Chalfont, PA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA). All

other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Pure Water Systems, Brea, CA).

2.3. In vitro evaluation of antioxidant properties

2.3.1. Preparation of the methanolic extracts

A fine dried powder (20 mesh; ~1 g) was extracted by stirring with 30 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 30 mL portion of methanol. The combined methanolic extracts were evaporated at 35ºC under reduced pressure, re-dissolved in methanol at a concentration of 10 mg/mL, and stored at 4 ºC for further use.

In vitro assays which have already been described by the authors (Guimarães *et al.*, 2010) were applied to evaluate the antioxidant activity of all the samples. Different concentrations of the extracts (4.00 to 0.03 mg/mL) were used to find EC_{50} values.

2.3.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The reaction mixture in each of the 96-wells consisted of one of the different concentrations of the extracts (30 µL) and aqueous methanolic solution (80:20, v/v, 270 µL) containing DPPH radicals (6×10^{-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.

2.3.3. 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. 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.

2.3.4. 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

158 zero time absorbance was measured at 470 nm using a 200-2004 spectrophotometer
159 (Analytikjena, Jena, Germany). A blank, devoid of β-carotene, was prepared for
160 background subtraction. β-Carotene bleaching inhibition was calculated using the
161 following equation: (β-carotene content after 2h of assay/initial β-carotene content) ×
162 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated
163 by interpolation from the graph of β-carotene bleaching inhibition percentage against
164 extract concentration. Trolox was used as standard.
165
166 *2.3.5. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances*
167 *(TBARS)*
168 Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and
169 homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a
170 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An
171 aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of
172 the extracts (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1
173 mM; 0.1 mL) at 37ºC for 1 h. The reaction was stopped by the addition of
174 trichloroacetic acid (28%, w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v,
175 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at
176 3000*g* (Centorion K24OR- 2003 refrigerated centrifuge) for 10 min to remove the
177 precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex
178 in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%)
179 was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] x 100%,
180 where A and B were the absorbance of the control and the compound solution,
181 respectively. The extract concentration providing 50% lipid peroxidation inhibition

182 (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract
183 concentration. Trolox was used as standard.
184
185 *2.4. Characterization in nutrients and phytochemicals*
186 *2.4.1. Phenolic compounds*
187 For total phenolics estimation an aliquot of the methanolic extract solution (1 mL) was
188 mixed with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10, v/v) and
189 sodium carbonate (75 g/L, 4 mL). The tubes were vortexed for 15 s and allowed to stand
190 for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm
191 (Wolfe *et al.*, 2003). Gallic acid was used to calculate the standard curve (0.0094-0.15
192 mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of
193 extract.
194
195 *2.4.2. Flavonoids*
196 For total flavonoids content determination, an aliquot (0.5 mL) of the methanolic extract
197 solution was mixed with distilled water (2 mL) and subsequently with $NaNO_2$ solution
198 (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to
199 stand further 6 min, thereafter, NaOH solution (4%, 2 mL) was added to the mixture.
200 Immediately, distilled water was added to bring the final volume to 5 mL. Then the
201 mixture was properly mixed and allowed to stand for 15 min. The intensity of pink
202 colour was measured at 510 nm (Jia *et al.*, 1999). (+)-Catechin was used to calculate the
203 standard curve (0.0045-0.29 mg/mL) and the results were expressed as mg of (+)-
204 chatechin equivalents (CE) per g of extract.
205

206 *2.4.3. Ascorbic acid*

207 A fine dried powder (20 mesh; 150 mg) was extracted with metaphosphoric acid (1%,
208 10 mL) for 45 min at room temperature and filtered through Whatman Nº 4 filter paper.
209 The filtrate (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance
210 was measured within 30 min at 515 nm (Klein and Perry, 1982). Content of ascorbic
211 acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid
212 (0.006-0.1 mg/mL), and the results were expressed as mg of ascorbic acid per 100 g of
213 dry weight.

214

215 *2.4.4. Tocopherols*

216 Tocopherols content was determined following a procedure previously optimized and
217 described by the authors (Barros *et al.*, 2010). BHT solution in *n*-hexane (10 mg/mL;
218 100 µL) and IS solution in *n*-hexane (tocol; 50 µg/mL; 400 µL) were added to the
219 sample prior to the extraction procedure. The samples (~500 mg) were homogenized
220 with methanol (4 mL) by vortex mixing (1 min). Subsequently, *n*-hexane (4 mL) was
221 added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2
222 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4,000*g*) and
223 the clear upper layer was carefully transferred to a vial. The sample was re-extracted
224 twice with *n*-hexane. The combined extracts were taken to dryness under a nitrogen
225 stream, redissolved in 2 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate,
226 filtered through 0.2 µm nylon filters from Whatman and transferred into a dark injection
227 vial. The equipment consisted of an integrated system with a Smartline 1000 pump
228 (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-
229 sampler (Jasco, Easton, MD) and an FP-2020 fluorescence detector (Jasco, Easton, MD)

programmed for excitation at 290 nm and emission at 330 nm. The column used was a normal-phase 250 mm × 4.6 mm i.d., 5 µm, Polyamide II, with a 10 mm × 4 mm i.d. guard column of the same material (YMC Waters, Dinslaken, Germany), operating at 30 ºC. 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 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 weight.

2.4.5. Liposoluble 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 (Nagata and Yamashita, 1992). 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 mg per 100 g of dry weight.

2.4.6. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as previously described by the authors (Guimarães *et al.*, 2010). Dried sample powder (1.0 g) was spiked with the melezitose as internal

254 standard (IS, 5 mg/ml), and was extracted with 40 mL of 80% aqueous ethanol at 80 ºC
255 for 30 min. The resulting suspension was centrifuged at 15,000*g* for 10 min. The
256 supernatant was concentrated at 60 ºC under reduced pressure (rotary evaporator Büchi
257 R-210) and defatted three times with 10 mL of ethyl ether, successively. After
258 concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5
259 mL and filtered through 0.2 µm nylon filters from Whatman. The equipment described
260 above was connected to a Smartline 2300 RI detector. Data were analysed using Clarity
261 DataApex 2.4 Software. The column used was a 250 mm × 4.6 mm i.d., 5 µm,
262 Eurospher 100-5 NH_2 with a 5 mm × 4mm i.d. guard column of the same material
263 (Knauer, Berlin, Germany), operating at 30 ºC in a 7971 R Grace oven. The mobile
264 phase was acetonitrile/deionized water, 7:3 (v/v) at a flow rate of 1 mL/min. Sugar
265 identification was made by comparing the relative retention times of sample peaks with
266 standards. Quantification was made by internal normalization of the chromatographic
267 peak area and the results are expressed in g per 100 g of dry weight.
268
269 *2.4.7. Fatty Acids*
270 Fat was extracted with petroleum ether in a Soxhlet apparatus. Fatty acids were
271 determined by gas chromatography with flame ionization detection (GC-FID) as
272 described previously by the authors (Guimarães *et al.*, 2010), and after the following
273 transesterification procedure: fatty acids (obtained after Soxhlet extraction) were
274 methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v/v/v), during at least
275 12 h in a water bath at 50 ºC and 160 rpm; then 3 mL of deionised water were added to
276 obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by
277 shaking in vortex, and the upper phase was passed through a micro-column of sodium

278 sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial
279 with Teflon, and filtered with 0.2 μm nylon filter from Whatman. The equipment was a
280 DANI model GC 1000 with a split/splitless injector, and a FID. The column used was a
281 30 m × 0.32 mm i.d., 0.25 μm, 50% cyanopropyl-methyl-50%
282 phenylmethylpolysiloxane (Macherey-Nagel, Düren, Germany). The oven temperature
283 program was as follows: the initial temperature of the column was 50 ºC, held for 2 min,
284 then a 10ºC/min ramp to 240 ºC and held for 11 min. The carrier gas (hydrogen) flow-
285 rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried
286 out at 250 ºC. Fatty acid identification was made by comparing the relative retention
287 times of FAME peaks from samples with standards. The results were recorded and
288 processed using CSW DataApex 1.7 software and expressed in relative percentage of
289 each fatty acid.
290
291 *2.5. Statistical analysis*
292 For each species, three samples were analysed and the assays were carried out in
293 triplicate. The results are expressed as mean values and standard deviation (SD). The
294 results were analyzed using one-way analysis of variance (ANOVA) followed by
295 Tukey's HSD Test with $\alpha = 0.05$ (different letters mean significant differences; the
296 letter a is attributed to the highest value). This treatment was carried out using SPSS v.
297 16.0 program.
298
299 **3. Results and discussion**
300 *3.1. In vitro evaluation of antioxidant properties*

A great number of aromatic, spicy, medicinal and other plants contain chemical compounds that exhibit antioxidant properties. Numerous studies were carried out on some of these plants, e.g. rosemary, sage, oregano, which resulted in a development of natural antioxidant formulations for food, cosmetic and other applications. However, scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine is still rather scarce. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals (Miliauskas *et al.*, 2004).

Herein, four different assays were used for the *in vitro* evaluation of the antioxidant properties of six medicinal plants widely used in Portugal as described in **Table 1**. The results of scavenging activity on DPPH radicals, reducing power, inhibition of β-carotene bleaching, and inhibition of lipid peroxidation in brain tissue homogenates, and also phenolic and flavonoids contents are shown in **Table 2.** *Rubus ulmifolius* 2 (flowers) proved to have the most promissory antioxidant activity (the lowest EC_{50} values, ranging from 0.02 to 0.07 mg/mL), with the highest phenolic (715.94 mg GAE/g extract) and flavonoids (624.76 mg CE/g extract; **Table 2**) contents which is according with its traditional use for gastrointestinal disorders and inflammatory processes. Moreover, EC_{50} values obtained in radical scavenging activity and reducing power of this sample (**Table 2**) were similar to those obtained for the standard trolox (0.04 and 0.03 mg/mL, respectively). Two different Italian group researchers reported the strong antioxidant properties of *Rubus ulmifolius* leaves attributing them to the activity of caffeic acid, ferulic acid and caffeic quinic esters as well as quercetin-3-O-glucuronide,

kaempferol-3-O-glucuronide (Dall'Acqua *et al.*, 2008), and to ellagic acid (Martini *et al.*, 2009).

Centaurea paniculata revealed the lowest antioxidant properties (the highest EC_{50} values, ranging from 3.62 to 0.74 mg/mL) which are compatible to its lower phenolics (35.87 mg GAE/g extract) and flavonoids (22.47 mg CE/g extract; **Table 2**) content.

Miliauskas *et al.* (2004) described a sample of *Matricaria recutita* (blossoms, full bloom stage) from Lithuania as a relatively weak antioxidant (44.7% of DPPH inhibition and 7.5 mg GAE/g extract for total phenolics). The sample analysed in the present study gave much higher phenolic content (139.62 mg GAE/g) and a DPPH radical scavenging activity of 62.35% at 1 mg/mL (**Table 2**). *Matricaria recutita* is one of the nine medicinal plants successfully used in STW 5 (Iberogast®) for treatment of gastrointestinal disorders (Schempp *et al.*, 2006). All the single extracts combined in STW 5 are of importance for the therapeutic effect, working in concert. This phytomedicine is prepared with ethanolic extracts and the mechanism of action is related to their antioxidant properties. In fact, functional gastrointestinal diseases, such as non-ulcer dyspepsia (NUD) and irritable bowel syndrome, are often initiated by or correlated to inflammatory processes, where oxidants such as ROS play a crucial role (Schempp *et al.*, 2006).

The phenolic content obtained in *Helichrysum stoechas* (184.42 mg GAE/g extract; **Table 2**) was higher than the content found in a sample from Turkey (94.16 mg GAE/g; Albayrak *et al.*, 2010). Both were methanolic extracts but the Turkish authors performed the extraction in a Soxhlet apparatus, which could destroy some phenolic compounds. Some authors reported that phenolic compounds are unstable and readily become non-antioxidative under heating and in the presence of antioxidants (Yen and

348 Hung, 2000; Barros *et al.*, 2007. Nevertheless, Carini *et al.* (2001) obtained a very high
349 antioxidant activity with a sample from Italy. They reported that the polar fraction
350 isolated from the flowering tops of *Helichrysum stoechas*, displays radical scavenging
351 properties, with potency comparable to that of Trolox, the water-soluble analogue of
352 vitamin E. The extract included approximately 50% of polyphenols and 4% of
353 kaempferol-3-*O*-glucoside, the more prominent component of the extract (Carini *et al.*,
354 2001).
355 *Castanea sativa* flowers (methanolic extract) gave highest DPPH scavenging activity
356 (0.07 mg/mL), reducing power (0.07 mg/mL), β-carotene bleaching inhibition (0.11
357 mg/mL), but lowest TBARS inhibition (0.03 mg/mL; **Table 2**) than a sample (aqueous
358 extract) also from Portugal (0.08 mg/mL, 0.09 mg/mL, 0.16 mg/mL and 0.01 mg/mL,
359 respectively; Barreira *et al.*, 2008). This is in agreement with the higher phenolics
360 (587.61 mg GAE/g extract) and flavonoids (165.45 mg CE/g; **Table 2**) content found in
361 our sample in comparison to the other sample (298.18 mg GAE/g and 159.56 mg CE/g,
362 respectively; Barreira *et al.*, 2008). This proves that the solvent used for phenolic
363 extraction has significant influence on the results. Herein, we decided to use methanol
364 according to previous results in experiments performed with different extraction
365 conditions to achieve the best procedure leading to highest contents in phenolics and
366 better antioxidant properties (Barros *et al.*, 2010). Furthermore, chemical and biological
367 diversity of aromatic and medicinal plants depend on such factors, as growth habitat,
368 climatic conditions, vegetation phase and genetic modifications (Miliauskas et al.,
369 2004).
370 Overall, among the medicinal plants analysed in the present study, *Rubus ulmifolius*
371 flowers revealed the highest antioxidant activity in all the tested assays.

372

373 *3.2. Nutrients and Phytochemicals*

374 Vitamins (tocopherols and ascorbic acid) and liposoluble pigments (carotenoids and
375 chlorophylls) contents of the six studied medicinal plants are given in **Table 3**. α-
376 Tocopherol was the major compound in all the samples, and δ-tocopherol was not
377 detected in *Centaurea paniculata* aerial parts. *Castanea sativa* flowers presented the
378 highest content of tocopherols (163.42 mg/ 100 g of dry weight), with the highest levels
379 of all the isoforms- α, β, δ, and γ-tocopherols. *Centaurea paniculata* and *Matricaria*
380 *recutita* aerial parts, *Rubus ulmifolius* flower buds and flowers revealed the lowest
381 contents without significant statistical differences (p<0.05).

382 Ascorbic acid was the most abundant vitamin in all the studied medicinal plants; *Rubus*
383 *ulmifolius* flower buds and flowers gave the highest levels (172.88 and 177.82 mg/100 g
384 dry weight, respectively and without significant statistical differences, p<0.05).

385 Carotenoids and chlorophylls were found in all the studied medicinal plants. ,
386 *Helichrysum stoechas* aerial parts revealed the highest concentration of β-carotene
387 (183.05 mg/100 g dry weight) and *Matricaria recutita* aerial parts gave the highest
388 concentration of chlorophylls a (170.26 mg/100 g) and b (56.37 mg/100 g). Lycopene
389 was found in very low amounts and only in *Rubus ulmifolius* flower buds and flowers,
390 and *Castanea sativa* flowers.

391 The levels of vitamins C and E, and β-carotene found in the medicinal plants would
392 make them suitable sources of these antioxidants that might be used commercially to
393 retard rancidity in fatty materials in food manufacturing, to reduce the effects of ageing
394 and to help to prevent oxidative-stress related diseases such as cancer and heart disease
395 (Dewick, 2002). In fact, synthetic antioxidants are being questioned while natural

antioxidants such as tocopherols, polyphenols and carotenoid pigments are having a greater relevance in the protection against lipid oxidation. Due to the role as a scavenger of free radicals, vitamins C and E are also believed to exert a protective role against various oxidative stress-related diseases such as heart disease, stroke, cancer, several neurodegenerative diseases and cataractogenesis (Halliwell, 1996). Also carotenoids have an important antioxidant role in the cellular protection against lipid peroxidation, thus preventing the risk of degenerative illnesses such as cancer, cardiovascular disease and macular degeneration. It also reduces the risk of cataracts and strengthens the immune system (Rao and Rao, 2007).

In summary, *Rubus ulmifolius* flowers revealed the highest contents in phenolics, flavonoids, ascorbic acid and lycopene, which is in agreement with its highest antioxidant activity. *Castanea sativa* flowers revealed the highest concentration of individual and total tocopherols. *Helichrysum stoechas* aerial parts gave the highest levels of β-carotene.

The effects of these compounds on the immune system could explain the empirical use of these plants to control inflammation, gastroenteritis and infectious diarrhea. In relation to sugar composition (**Table 4**) *Castanea sativa* flowers and *Trifolium angustifolium* aerial parts gave fructose as the main sugar, while sucrose predominated in *Centaurea paniculata, Helichrysum stoechas* and *Matricaria recutita* aerial parts, and glucose was the main sugar in *Rubus ulmifolius* flower buds and flowers. *Castanea sativa* flowers revealed the highest total sugars content (11.91 g/100 g dry weight), with the highest levels of fructose (5.05 g/100 g) and glucose (4.62 g/100 g). Otherwise, *Helichrysum stoechas* aerial parts showed the lowest levels in total sugars (3.87 g/100

g). Some of the identified sugars, mostly the reducing sugars fructose, glucose and raffinose could also contribute to the antioxidant activity observed in the studied plants. Overall, *Castanea sativa* flowers revealed the highest concentration of total sugars, including the reducing sugars glucose and fructose.

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 medicinal plants are shown in **Table 5**. Twenty three fatty acids were identified and quantified. The major fatty acids found were linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) and contributing to the prevalence of PUFA. Palmitic acid (C16:0) was also a main fatty acid in all the studied plants, while arachidic acid (C20:0) and behenic acid (C22:0) were abundant in *Castanea sativa* flowers, contributing to the highest SFA content found in this sample (51%). Oleic acid (C18:1n9) was abundant in *Centaurea paniculata* aerial parts contributing to the increase of its MUFA content (18.5%). *Matricaria recutita* aerial parts revealed the highest PUFA contents (68.9%) with the highest levels of linoleic acid (44.8%).

α-Linolenic and linoleic acids, as also high ratios of PUFA/SFA (> 0.45) and low n-6/n-3 fatty acids ratios (< 4.0) can decrease the total amount of fat in blood (cholesterol), reducing the risk of cancer, cardiovascular, inflammatory and autoimmune diseases (HMSO, 1994; Kanu *et al.*, 2007). Once again these results are according to folk recommendations. For instance, it is considered that chestnut catkins decoctions, drunk for nine days, can lower blood cholesterol levels, and several informants pointed out that German chamomile infusion may help reduce cholesterol.

Overall, *Matricaria recutita* aerial parts revealed the highest PUFA levels, including linoleic acid.

In conclusion, the studied medicinal plants revealed interesting antioxidant properties, nutrients and phytochemicals such as phenolics, flavonoids, vitamins, carotenoids, sugars, and fatty acids that could provide scientific evidence for some folk uses in the treatment of diseases related to the production of ROS and oxidative stress, but further experiments are required to explore the mechanisms of action. Traditional medicines, whose knowledge and practices have been orally transmitted over the centuries, are important approaches for discovering therapeutic molecules and compounds. This study provides information useful not only to chemical analysis, activity assays and standardization of phytochemical composition of wild species traditionally used, but to researchers in phytopharmacology, phytotherapy and phytotoxicology as well. As far as we know this is the first report on chemical characterization of six medicinal plants from the Portuguese folk medicine: *Castanea sativa, Centaurea paniculata, Helichrysum stoechas*, *Matricaria recutita, Rubus ulmifollius* and *Trifolium angustifolium*.

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Table 1. Medicinal uses of the studied plants reported in Portuguese ethnobotanical studies conducted in the north-eastern region.

Scientific name	English name	Local name	Medicinal uses
Castanea sativa	Chestnut flower	Flor-de-castanheiro	Cold, cough, diarrhea, cholesterol
Centaurea paniculata	starthistles, knapweeds	Escalabriosa	Inflammation
Helichrysum stoechas	Shrubby everlasting	Douradinha	Cold, bronchitis, fever
Matricaria recutita	German chamomile	Maçiela	Respiratory and digestive systems, inflammation
Rubus ulmifolius	Elm-leaved blackberry;	Silva, silva-gravanceira	Stomachache or abdmominal pane diarrhea
Trifolium angustifolium	Narrow clover	Rabo-de-gato	Diarrhea

Table 2. Extraction yields, antioxidant activity (EC_{50} values, mg/mL) of six medicinal plants, and composition in phenolics and flavonoids (mean ± SD; n=3). In each row different letters mean significant differences ($p<0.05$).

	Castanea sativa	*Centaurea paniculata*	*Helichrysum stoechas*	*Matricaria recutita*	*Rubus ulmifolius* 1	*Rubus ulmifolius* 2	*Trifolium angustifolium*
η (%)	38.46 ± 0.94	40.32 ± 1.18	17.13 ± 0.23	16.09 ± 1.01	38.35 ± 0.79	39.52 ± 2.96	15.74 ± 0.89
DPPH scavenging activity	0.07 ± 0.00 fe	3.62 ± 0.13 a	0.52 ± 0.00 d	0.80 ± 0.05 b	0.12 ± 0.00 e	0.04 ± 0.00 f	0.70 ± 0.04 c
Reducing power	0.07 ± 0.00 f	1.33 ± 0.02 a	0.14 ± 0.01 d	0.21 ± 0.02 c	0.10 ± 0.00 e	0.04 ± 0.00 g	0.45 ± 0.01 b
β-carotene bleaching inhibition	0.11 ± 0.01 e	1.25 ± 0.09 a	0.25 ± 0.08 d	0.66 ± 0.02 c	0.21 ± 0.00 d	0.07 ± 0.00 e	0.91 ± 0.11 b
TBARS inhibition	0.03 ± 0.00 f	0.74 ± 0.03 a	0.13 ± 0.00 d	0.18 ± 0.00 c	0.07 ± 0.00 e	0.02 ± 0.00 f	0.29 ± 0.01 b
Phenolics (mg GAE/g extract)	587.61 ± 3.91 b	35.87 ± 1.79 g	184.42 ± 0.35 d	139.62 ± 3.02 e	257.89 ± 3.28 c	715.94 ± 28.55 a	95.61 ± 1.48 f
Flavonoids (mg CE/g extract)	165.45 ± 2.48 b	22.47 ± 0.29 d	34.75 ± 0.83 d	126.82 ± 3.95 c	172.45 ± 3.42 b	624.76 ± 1.18 a	26.78 ± 3.10 d

EC_{50} values for the standard trolox: 43 μg/ml (DPPH scavenging activity); 30 μg/ml (Reducing power); 3 μg/ml (β-carotene bleaching inhibition) and 4 μg/ml (TBARS inhibition).

Table 3. Composition in vitamins and liposoluble pigments (mg/100 g dry weight) of six medicinal plants (mean ± SD; n=3). In each row different letters mean significant differences ($p<0.05$).

	Castanea sativa	*Centaurea paniculata*	*Helichrysum stoechas*	*Matricaria recutita*	*Rubus ulmifolius* 1	*Rubus ulmifolius* 2	*Trifolium angustifolium*
α-tocopherol	124.64 ± 2.28 a	8.54 ± 0.46 dc	66.43 ± 3.61 b	3.52 ± 0.30 e	5.97 ± 0.20 de	5.84 ± 0.01 de	12.73 ± 1.14 c
β-tocopherol	3.62 ± 0.20 a	0.25 ± 0.05 c	1.70 ± 0.23 b	0.18 ± 0.02 c	0.28 ± 0.10 c	0.18 ± 0.00 c	0.60 ± 0.06 c
γ-tocopherol	20.26 ± 0.94 a	1.82 ± 0.15 d	4.20 ± 0.01 c	2.44 ± 0.06 d	4.05 ± 0.01 c	2.55 ± 0.01 d	5.41 ± 0.62 b
δ-tocopherol	14.90 ± 0.80 a	nd	1.28 ± 0.01 b	1.20 ± 0.23 cb	1.98 ± 0.01 b	1.28 ± 0.05 b	0.39 ± 0.02 d
Total tocopherols	163.42 ± 4.22 a	10.60 ± 0.66 d	73.61 ± 3.85 b	7.35 ± 0.03 d	12.28 ± 0.21 d	9.86 ± 0.05 d	19.13 ± 2.00 c
Ascorbic acid	163.48 ± 8.20 b	40.45 ± 1.15 d	157.07 ± 10.48 b	61.57 ± 0.81 c	172.88 ± 1.89 a	177.82 ± 6.97 a	163.80 ± 2.48 b
β-carotene	43.53 ± 0.08 e	69.25 ± 0.08 d	183.05 ± 0.75 a	127.68 ± 0.29 c	34.22 ± 0.28 g	38.77 ± 0.15 f	160.69 ± 1.13 b
Lycopene	0.05 ± 0.00 b	nd	nd	nd	0.01 ± 0.00 c	0.07 ± 0.00 a	nd
Chlorophyll a	1.06 ± 0.00 e	1.23 ± 0.00 d	2.72 ± 0.00 c	170.26 ± 0.08 a	0.63 ± 0.00 f	1.09 ± 0.00 e	4.75 ± 0.00 b
Chlorophyll b	0.46 ± 0.00 d	0.35 ± 0.00 d	1.02 ± 0.00 c	56.37 ± 0.36 a	0.27 ± 0.00 d	0.59 ± 0.00 d	1.77 ± 0.00 b

nd- not detected.

Table 4. Composition in sugars (g/100 g of dry weight) of six medicinal plants (mean ± SD; n=3). In each row, different letters mean significant differences ($p<0.05$).

	Castanea sativa	*Centaurea paniculata*	*Helichrysum stoechas*	*Matricaria recutita*	*Rubus Ulmifolius* 1	*Rubus Ulmifolius* 2	*Trifolium angustifolium*
Fructose	5.05 ± 0.66 a	0.89 ± 0.01 c	1.02 ± 0.04 c	1.64 ± 0.04 c	3.33 ± 0.01 b	1.66 ± 0.21 c	3.48 ± 0.31 b
Glucose	4.62 ± 0.59 a	0.50 ± 0.01 d	0.59 ± 0.02 d	0.89 ± 0.01 d	3.71 ± 0.03 b	2.23 ± 0.19 c	1.15 ± 0.10 d
Sucrose	1.65 ± 0.14 b	2.28 ± 0.04 a	1.84 ± 0.09 b	2.53 ± 0.10 a	0.85 ± 0.01 d	1.34 ± 0.15 c	1.21 ± 0.13 c
Trehalose	0.44 ± 0.04 b	0.44 ± 0.00 b	0.42 ± 0.05 b	0.84 ± 0.13 a	0.84 ± 0.01 a	0.72 ± 0.04 a	0.17 ± 0.01 c
Raffinose	0.15 ± 0.05 dc	0.58 ± 0.00 a	nd	0.19 ± 0.00 c	0.09 ± 0.01 d	0.10 ± 0.01 d	0.51 ± 0.01 b
Total sugars	11.91 ± 1.49 a	4.69 ± 0.05 de	3.87 ± 0.07 e	6.08 ± 0.19 dc	8.83 ± 0.02 b	6.03 ± 0.59 dc	6.52 ± 0.63 c

nd- not detected

Table 5. Composition in fatty acids of six medicinal plants (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

	Castanea sativa	*Centaurea paniculata*	*Helichrysum stoechas*	*Matricaria recutita*	*Rubus ulmifolius* 1	*Rubus ulmifolius* 2	*Trifolium angustifolium*
C6:0	0.16 ± 0.01	0.11 ± 0.00	0.15 ± 0.01	0.24 ± 0.03	0.31 ± 0.00	0.09 ± 0.01	2.00 ± 0.13
C8:0	0.51 ± 0.05	0.11 ± 0.01	2.42 ± 0.13	0.22 ± 0.03	0.10 ± 0.00	0.08 ± 0.00	0.79 ± 0.14
C10:0	0.32 ± 0.01	0.11 ± 0.00	1.71 ± 0.12	0.23 ± 0.02	0.08 ± 0.00	0.07 ± 0.00	0.24 ± 0.06
C11:0	nd	nd	0.52 ± 0.13	0.10 ± 0.00	nd	nd	nd
C12:0	0.33 ± 0.01	1.65 ± 0.21	7.58 ± 0.05	0.20 ± 0.01	0.62 ± 0.03	0.49 ± 0.00	2.28 ± 0.16
C13:0	0.02 ± 0.00	0.02 ± 0.00	0.17 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C14:0	1.06 ± 0.05	1.02 ± 0.08	7.91 ± 0.70	1.34 ± 0.03	1.12 ± 0.02	0.47 ± 0.05	4.84 ± 0.25
C14:1	0.18 ± 0.00	0.23 ± 0.00	0.15 ± 0.00	0.17 ± 0.01	0.07 ± 0.00	0.06 ± 0.01	0.15 ± 0.01
C15:0	0.30 ± 0.01	0.31 ± 0.01	0.63 ± 0.01	0.26 ± 0.01	0.19 ± 0.03	0.16 ± 0.01	0.33 ± 0.02
C16:0	14.40 ± 0.82	17.46 ± 0.37	13.24 ± 0.16	13.56 ± 0.22	11.99 ± 0.03	12.05 ± 0.51	15.42 ± 0.72
C16:1	0.19 ± 0.03	2.24 ± 0.14	0.24 ± 0.02	0.23 ± 0.00	0.22 ± 0.00	0.15 ± 0.02	0.27 ± 0.03
C17:0	0.47 ± 0.02	0.45 ± 0.07	0.61 ± 0.05	0.40 ± 0.00	0.34 ± 0.01	0.33 ± 0.05	0.44 ± 0.02
C18:0	4.82 ± 0.11	3.64 ± 0.22	2.60 ± 0.14	2.80 ± 0.01	3.08 ± 0.02	2.96 ± 0.07	3.22 ± 0.03
C18:1n9c	5.84 ± 0.06	15.76 ± 1.06	6.15 ± 0.79	7.99 ± 0.02	4.30 ± 0.00	3.04 ± 0.09	6.11 ± 1.28
C18:2n6c	20.88 ± 0.89	34.92 ± 0.87	25.67 ± 0.08	44.83 ± 0.04	16.02 ± 0.07	14.98 ± 0.13	20.15 ± 0.40
C18:3n3	21.24 ± 0.14	14.71 ± 0.83	22.79 ± 1.86	22.89 ± 0.12	39.56 ± 0.03	38.04 ± 0.08	34.72 ± 1.13
C20:0	11.15 ± 0.52	2.30 ± 0.24	1.75 ± 0.13	0.65 ± 0.02	6.14 ± 0.10	7.77 ± 0.59	2.55 ± 0.05
C20:1c	0.08 ± 0.00	0.27 ± 0.01	0.44 ± 0.03	0.03 ±0.00	0.02 ± 0.00	0.03 ± 0.00	0.24 ± 0.06
C20:2c	0.06 ± 0.00	0.05 ± 0.00	0.29 ± 0.04	0.06 ± 0.00	0.12 ± 0.01	0.17 ± 0.00	0.12 ± 0.03
C20:3n3+C21:0	0.55 ± 0.00	0.05 ± 0.01	0.23 ± 0.02	1.17 ± 0.03	0.54 ± 0.05	0.55 ± 0.09	0.37 ± 0.03
C22:0	11.54 ± 0.78	2.93 ± 0.61	2.06 ± 0.04	0.68 ± 0.05	8.69 ± 0.02	11.41 ± 0.77	2.36 ± 0.23
C23:0	nd	nd	nd	nd	0.27 ± 0.05	0.08 ± 0.02	0.17 ± 0.01
C24:0	5.90 ± 0.41	1.68 ± 0.13	2.68 ± 0.38	1.93 ± 0.13	6.20 ± 0.16	7.02 ± 0.45	3.21 ± 01.0
Total SFA	51.00 ± 0.72 a	31.78 ± 0.25 e	44.04 ± 0.89 b	22.63 ± 0.16 f	39.16 ± 0.18 c	42.99 ± 0.73 b	37.86 ± 0.66 d
Total MUFA	6.28 ± 0.03 cd	18.49 ± 2.21 a	6.98 ± 0.84 cb	8.42 ± 0.03 b	4.61 ± 0.01 ed	3.28 ± 0.85 e	6.77 ± 1.33 cb
Total PUFA	42.67 ± 0.75 e	49.68 ± 1.97 d	48.69 ± 1.77 d	68.89 ± 0.19 a	56.11 ± 0.16 cb	53.56 ± 0.13 c	55.24 ± 0.71 cb
PUFA/SFA	0.84 ± 0.03 f	1.56 ± 0.05 b	1.11 ± 0.06 e	3.04 ± 0.03 a	1.43 ± 0.01 c	1.25 ± 0.02 d	1.46 ± 0.01 c
n-6/n-3	0.98 ± 0.05 c	2.43 ± 0.53 a	1.13 ± 0.10 c	1.96 ± 0.01 b	0.40 ± 0.00 d	0.39 ± 0.00 d	0.58 ± 0.03 d

nd- not detected. Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecanoic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosanoic acid (C20:1c); Eicosadienoic acid (C20:2c); Eicosatrienoic acid + Heneicosanoic acid (C20:3n3+C21:0); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).