Comparing the composition and bioactivity of *Crataegus monogyna* flowers and fruits used in folk medicine

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Running title: Comparing the composition and bioactivity of *Crataegus monogyna*

ABSTRACT

Introduction- Studying local plant foods is of particular interest as they often contain high amounts of bioactive compounds. Furthermore, their nutritional and medicinal impact must be documented and supported with scientific studies. *Crataegus monogyna* is an example of "functional food" traditionally used all over South European countries.

Objective- A complete chemical and bioactive characterization of flower buds, flowers, unripe, ripened and over ripened fruits was performed.

Methodology- Chemical characterization included determination of proteins, fats, ash, and carbohydrates, particularly sugars by HPLC-RI, fatty acids by GC-FID, tocopherols by HPLC-fluorescence, phenolics, flavonoids, β-carotene and ascorbic acid, by spectrophotometric techniques. Bioactivity was evaluated through screening of antioxidant properties: radical scavenging effects, reducing power, and inhibition of lipid peroxidation.

Results- Flowers revealed the highest tocopherols and ascorbic acid contents, as also the best n-6/n-3 fatty acids ratio. Over ripened fruits showed the highest levels of carbohydrates, sugars and SFA. Unripe fruits presented the highest PUFA contents with the best PUFA/SFA ratio, as also the highest levels of phenolics and the most promising antioxidant properties (EC_{50} < 20.83 μg/ml; even better than trolox).

Conclusion- This study shows the potential of different parts of *Crataegus monogyna* as sources of several compounds, including nutrients and nutraceuticals. Moreover, it supports the documented nutritional and medicinal impact of this species.

Keywords: Crataegus monogyna; Flowers and fruits; Composition; Bioactivity; Portuguese Ethnoflora

Introduction

The human body produces reactive oxygen species (ROS) during normal metabolism. In conditions of oxidative stress, the production of prooxidants exceed the antioxidant capacity causing oxidation of biomolecules such as DNA, leading to cellular damage and contributing to several disorders of the circulatory and respiratory systems and to certain chronic disease states such as cancer. Oxidative stress can be attenuated or perhaps reversed by diets containing fruits, vegetables and herbs that have antioxidant activities (Mizaton *et al.*, 2009) due to their high content in bioactives, such as polyphenols (especially flavonoids), vitamins and unsaturated fatty acids (UFA). Studying local plant used as both food and medicine is of particular interest as they often contain higher amounts of those bioactive compounds than highly cultivated food plants, especially ones, which have been under cultivation for many generations (Heinrich *et al.*, 2005). Furthermore, the nutritional impact of locally grown and consumed wild or semi-wild plants must be documented and supported with scientific studies.

An ethnobotanical survey conducted in north-eastern Portugal (Carvalho, 2005) reported that 20% of the catalogued species were quoted as having interesting food-medicine linkages. Common hawthorn, (port:“espinheiro, escaramunheiro”), *Crataegus monogyna* Jacq., is one of the species that is highly recommended in folk medicine and the “berries” are compulsively consumed by shepherds, hunters and children, because they are considered to be “healthy” and nutritious. In several Portuguese regions, hawthorn is regarded as especially important in the management and prevention of age-related diseases (for instance, cardiovascular disease, atherosclerosis, arthritis, and hypertension). It also cures colds, other upper respiratory infection, bronchitis,

pneumonia, and it is used as a tranquilizer and to control cellulite, obesity and menopause disturbances. Women used to gather the autumnal ripened fruits and give them to children as they believe it is a good vitamin supplier (Camejo-Rodrigues *et al.*, 2003; Novais *et al.*, 2004; Carvalho, 2005; Neves *et al.*, 2009). Moreover, some other studies document and confirm the medicinal use of hawthorn flowers and fruits in the treatment of chronic heart failure, high blood pressure, arrhythmia, and various digestive ailments, as well in geriatric and arteriosclerosis remedies. Fruits are also reported as foodstuff (canned fruit, jam, jelly, drink, and wine) (Pardo de Santayana *et al.*, 2007; Bernatonienė *et al.*, 2008; Tadić *et al.*, 2008; Signorini *et al.*, 2009).

Considering that chain radical reactions are involved in the oxidation of lipids and other biomolecules (eg. DNA), plants can be promising in the development of bioactive ingredients for functional foods, nutraceuticals, medicinal preparations and other applications (Romero-Jiménez *et al.*, 2005). Therefore, the present study includes data regarding a complete chemical and bioactive characterization of *Crataegus monogyna* aerial parts, gathered in Bragança, a Portuguese north-eastern region. Chemical characterization included determination of protein, fat, ash, carbohydrates, free sugars, fatty acids, tocopherols, ascorbic acid, β-carotene, phenolics and flavonoids; Bioactivity evaluation included determination of antioxidant properties by radical scavenging capacity, reducing power and lipid peroxidation inhibition assays. Moreover, the nutritional and medicinal impact of this plant will be scientifically documented.

Experimental

Samples

87 The material for analysis was gathered in sequence, during 2009 spring, summer and
88 autumn, synchronized with the growth condition of buds, flowers and fruits, according
89 to different gathering practices, folk pharmacopoeia and local edible uses reported in the
90 studied area (Bragança, North-eastern Portugal). Five different samples were
91 considered: flower buds with top young leaves (corymbs); flowers during anthesis
92 (flower fully opened and functional) plus few expanded leaves attached at the base of
93 the inflorescence peduncle; unripe fruits corresponding to flower senescence and stand
94 out of the green pomaceous (berry-like) immature fruit; ripened fruits i.e. red pomes in
95 late summer; over ripened fruits i.e. dark red, fleshy, sweet, chewy and coarse-textured
96 pomes in late autumn (**Figure 1**).
97 Morphological key characters from the Flora Iberica (Castroviejo, 2001) were used for
98 plant identification. Voucher specimens are deposited in the Escola Superior Agrária de
99 Bragança herbarium (BRESA). Each sample was lyophilized (Ly-8-FM-ULE, Snijders,
100 Holland) and kept in the best conditions for subsequent use.
101
102 **Standards and Reagents**
103 Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from
104 Lab-Scan (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard
105 mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as
106 also other individual fatty acid isomers, ascorbic acid, tocopherols and sugars standards,
107 trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-
108 catechin. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-
109 Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,
110 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).

Composition

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. Total energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein +g carbohydrates) + 9 × (g fat).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described previously by us (Barros *et al.*, 2010a). Dried sample powder (1.0 g) was spiked with 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 (rotary evaporator Büchi R-210) and defatted three times with 10 mL of ethyl ether. 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. The HPLC 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 Eurospher 100-5 NH_2 column (4.6 × 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 were expressed in g per 100 g of dry weight.

Fatty Acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID) as described previously by the authors (Barros *et al.*, 2010a), and after the following trans-esterification procedure: fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid 95%:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 ºC and 160 rpm; to obtain phase separation 3 mL of deionised water were added; the fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 mL of diethyl ether, and the upper phase was passed through a micro-column of anhydrous sodium sulphate, in order to eliminate the water. The sample was recovered after filtration with 0.2 µm nylon filter from Whatman. The fatty acid profile was analyzed in a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 10ºC/min ramp to 240 ºC and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried

159 out at 250 ºC. For each analysis 1 µL of the sample was injected in GC. Fatty acid
160 identification was made by comparing the relative retention times of FAME peaks from
161 samples with standards. The results were recorded and processed using CSW 1.7
162 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.
163
164 **Tocopherols.** Tocopherols content was determined following a procedure previously
165 optimized and described by Barros *et al.* (2010b). BHT solution in hexane (10 mg/mL;
166 100 µL) and IS solution in hexane (tocol; 50 µg/mL; 400 µL) were added to the sample
167 prior to the extraction procedure. The samples (500 mg) were homogenized with
168 methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added
169 and again vortex mixed for 1 min. Saturated NaCl aqueous solution (2 mL) was added,
170 the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the clear upper
171 layer was carefully transferred to a vial. The sample was re-extracted twice with hexane.
172 The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2
173 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2 µm
174 nylon filters from Whatman, transferred into a dark injection vial and analysed by the
175 HPLC system described above connected to a fluorescence detector (FP-2020; Jasco)
176 programmed for excitation at 290 nm and emission at 330 nm. The chromatographic
177 separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column
178 from YMC Waters operating at 30ºC. The mobile phase used was a mixture of *n*-hexane
179 and ethyl acetate (7:3, v/v) at a flow rate of 1 mL/min, and the injection volume was 20
180 µL. The compounds were identified by chromatographic comparisons with authentic
181 standards. Quantification was based on the fluorescence signal response, using the

internal standard method. Tocopherol contents in the samples were expressed in mg per 100 g of dry weight.

Ascorbic acid. Ascorbic acid 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 ascorbic acid 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.

β-Carotene. β-Carotene was 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) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$, and further expressed in mg per 100 g of dry weight.

Bioactivity

General. *In vitro* assays already described by the authors elsewhere (Barros *et al.*, 2010b), were applied to evaluate the antioxidant activity of the samples. Different concentrations of the extracts (7.8–500 µg/mL) were used to find EC_{50} values. The

206 extractions were performed using a fine dried powder (20 mesh; ~1g) stirring with 50
207 mL of methanol at 25 ºC and 150 rpm for 1 h, and filtered through Whatman No. 4
208 paper. The residue was then extracted with one additional 50 mL portion of methanol.
209 The combined methanolic extracts were evaporated at 35ºC under reduced pressure, re-
210 dissolved in methanol at 10 mg/mL, and stored at 4 ºC for further use.
211
212 **DPPH radical-scavenging activity.** This methodology was performed using an
213 ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each
214 one of the 96-wells consisted of one of the different concentrations of the extracts (30
215 μL) and aqueous methanolic solution (8:2 v/v, 270 μL) containing DPPH radicals
216 (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of
217 the DPPH radical was determined by measuring the absorption at 515 nm. The radical
218 scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using
219 the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the
220 solution when the sample extract has been added at a particular level, and A_{DPPH} is the
221 absorbance of the DPPH solution. The extract concentration providing 50% of radicals
222 scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against
223 extract concentration. Trolox was used as standard.
224
225 **Reducing power.** This methodology was performed using the Microplate Reader
226 described above. The different concentrations of the extracts (0.5 mL) were mixed with
227 sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1%
228 w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid
229 (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.

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 chloroform evaporation at 40ºC under reduced pressure, 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. A blank, devoid of β-carotene, was prepared for background subtraction. β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

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 3000*g* 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 37 ºC 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 3000*g* 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] × 100%, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract concentration. Trolox was used as standard.

Phenolics and flavonoids

Phenolics were estimated based on procedures described by Wolfe *et al.* (2003) with some modifications. An aliquot of the extract solution (1 mL) was mixed with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Flavonoids were determined using the method of Jia *et al.* (1999), with some modifications. An aliquot (0.5 mL) of the extract solution was mixed with distilled

water (2 mL) and subsequently with $NaNO_2$ solution (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 mL) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. The mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.0156-1.0 mM) and the results were expressed as mg of (+)-catechin equivalents (CEs) per g of extract.

Statistical analysis

For each one of the samples the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 16.0 program.

Results and discussion

Composition and bioactivity of different parts of *Crataegus monogyna* traditionally used for nutritional and medicinal proposals (flower buds and flowers, unripe, ripened and over ripened fruits) were compared.

Composition

Wild food used to be much praised and *Crataegus monogyna* is one of the species most mentioned in several European regions. Pickled flower buds are used as a pungent relish in various dishes (Signorini *et al.*, 2007). Flower buds and top young leaves are eaten in salads, seasoned with olive oil, lemon or vinegar. The fruits are eaten raw, dried, or

made in jam, marmalade and liquors (Carvalho, 2005; Lentini and Venza, 2007; Pardo de Santayana *et al.*, 2007).

The results of the nutrients composition and energetic value (expressed on dry weight basis) obtained for the *Crataegus monogyna* parts are shown in **Table 1**. Unripe fruits revealed the highest moisture content (76.81 g/100 g, dw), while over ripened fruits showed the lowest contents (59.76 g/100 g, dw). Carbohydrates, calculated by difference, were the most abundant macronutrients, particularly for fruits (> 91.2 g/100 g, dw). In view of their carbohydrates content these wild fruits represented an important contribution to local daily diets, especially during autumn and early winter. Protein and fat contents were higher in flowers than in fruits, which is an expected result considering that the flowering process stimulates plants growth rate, associated to the synthesis of peptides and proteins. Moreover protein is a major component of florigen (flowering hormone) which participate in floral induction. Flower buds revealed the highest values of protein. Fat was the less abundant macronutrient being lower than 3.6 g/100 g dw for flowers and 0.8 g/100 g dw for fruits; over ripened fruits revealed the lowest levels. On the basis of the proximate analysis, it can be calculated that a dry portion of 100 g of flower buds or flowers assures, on average, 387 Kcal, a lower value than the energy supplied by fruits (392 Kcal). Ash falls between proteins and fat contents, being more abundant in flowers (7.25 g/100 g, dw), and less abundant in unripe fruits (2.39 g/100 g, dw). Özcan *et al.* (2005) reported similar values for hawthorn fruits from Turkey: moisture 64.26%, protein 2.48%, fat 0.87% and ash 2.28%. Nevertheless, this manuscript does not specify the hawthorn species used.

Crataegus monogyna showed fructose, glucose, sucrose and trehalose as main sugars (**Table 1**). The present study describes, for the first time, sugars composition in this

plant, including flower buds, flowers and fruits in different stages of maturity. Glucose predominates in all the studied parts. Over ripened fruits revealed the highest total sugars content, with highest levels of fructose and glucose (reducing sugars), which is in agreement with their sweeter taste, and with informants' perception of being the best growth stage to consume them raw. Furthermore, sugar contents in ripened and over ripened fruits are a significant part of carbohydrates. Unripe fruits showed the lowest levels in total sugars (2.21 g/100 g, dw). The green fruits' content of sugars, phenolics and flavonoids are strategies to discourage herbivory, avoiding early dispersal of immature seeds.

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 parts of *Crataegus monogyna* are shown in **Table 2**.The major fatty acid found was tricosanoic acid (C23:0) with the exception of unripe fruits where linoleic acid (C18:2) predominated (58.5%), contributing to the prevalence of PUFA. Flower buds and flowers also revealed significant amounts of α-linolenic acid (C18:3). The last two mentioned fatty acids must be obtained by the diet and originate omega-3 and omega-6 fatty acids, respectively. In all the parts, PUFA predominated over MUFA due to the abundance of C18:2 and C18:3. Palmitic acid was the second SFA most abundant in the samples. Nineteen fatty acids were identified and quantified.

The ratios of PUFA/SFA and n-6/n-3 are nutritional indexes widely used to evaluate the nutritional value of fat for human diet. Current nutritional recommendations are that PUFA/SFA ratios in human diet should be above 0.45, and n-6/n-3 ratios should not exceed 4.0 (HMSO, 1994). All the parts with the exception of ripened fruits revealed

PUFA/SFA ratios higher than 0.45 (**Table 2**); the best values were given by unripe fruits (2.59). The n-6/n-3 ratios were always lower than 4.0, unless in unripe fruits; the best values were obtained for flowers (0.48). Low ratios of PUFA/SFA and high levels of cholesterol have been considered as major risk factors for cardiovascular diseases, which are among the most important causes of human mortality in developed countries. Moreover, a very high n-6/n-3 ratio (15-17) not only favours the development of cardiovascular diseases, but also cancer, inflammatory and autoimmune diseases. In addition, fats presenting low PUFA/SFA ratio are considered unfavourable, because they may induce and increase cholesterolaemia (Prates *et al.*, 2007). Therefore, according to the obtained results, *Crataegus monogyna* should be considered healthy food. As far as we know, nothing has been reported on fatty acid composition of flowers and fruits of this wild plant. Nevertheless, there is a report on fatty acids composition of epicuticular wax extracts from *Crataegus monogyna* flowers (Griffiths *et al.*, 2000).

Vitamins (tocopherols and ascorbic acid) and β-carotene contents were also determined and the results are given in **Table 3**. The four isoforms of tocopherols were detected, being α-tocopherol the major compound in all the parts. Flowers presented the highest content of tocopherols (159.84 mg/ 100 g of dry weight) while unripe fruits revealed the lowest content (21.75 mg/100 g). Griffiths *et al.* (2000) also described the alpha isoform as the major tocopherol in epicuticular wax extracts from *Crataegus monogyna* flowers. Medical literature provides a wealth of examples of the efficacy of α-tocopherol in preventing or arresting free-radical induced diseases such as many types of cancer, atherosclerosis and other circulatory diseases, arthritis, cataract formation, senile dementia (Alzheimer type), respiratory diseases induced by pollution, and the aging itself (Horwitt, 1991; Packer, 1991). Another important antioxidant is ascorbic acid,

374 being predominant in flower samples (408.37 mg/100 g; **Table 3**). Such results may be
375 explained by the presence of some expanded leaves attached in corymbs' base (**Figure**
376 **1**). Sampling took into account informants' gathering perceptions and they believe that
377 the most suitable material must include the inflorescences and tops leaves. Therefore,
378 *Crataegus monogyna* flowers with top leaves are considerable sources of these two
379 antioxidant vitamins. β-carotene was found in high amounts in over ripened fruits
380 (94.15 mg/100 g dry weight; **Table 3**).
381
382 **Bioactivity**
383 *Crategus monogyna* is also of great importance in folk medicine. Flower buds, flowers
384 and top leaves are used to prepare infusions and decoctions that treat affections of the
385 respiratory and circulatory systems, nervous system disorders (such as migraines,
386 confusion, irritability and memory loss) and insomnia (Camejo-Rodrigues *et al.*, 2003;
387 Novais et al., 2004; Carvalho, 2005; Neves *et al.*, 2009). The juice of smashed immature
388 fruits or from ripened fruits (depending on the informants' traditional knowledge) is a
389 topical preparation for skin application that relieves pain and stiffness, such as from sore
390 muscles or from arthritis. Decoctions of fresh and dried fruits are used as diuretics
391 (Carvalho, 2005).
392 It was not observed any correlation between extraction yields and phenolic contents
393 (**Table 4**). Phenolics were the major antioxidant components (247.03-701.65 mg GAE/g
394 of extract); unripe fruits revealed the highest content in phenolics and flavonoids. The
395 amount of phenolics found in the analysed samples were higher than the ones found in
396 hawthorn berries ethanolic extracts (according to European Pharmacopoeia 6.0,
397 hawthorn berries consist of the dried pomes of *Crataegus monogyna* Jacq. and

398 *Crataegus oxyacantha* L. or their mixture): 35.4 mg GAE/g (Tadić *et al.*, 2008).
399 Froehlicher *et al.* (2009) also reported the phenolic contents in fresh fruit, dried fruit,
400 flowering tops (flowers with young leaves) and flowers of *C. monogyna* from France,
401 but the results were expressed in mg GAE/100 g of dry weigh.
402 The antioxidant activity of *Crataegus monogyna* was evaluated by four in vitro
403 chemical and biochemical assays using animal cells: scavenging effects on DPPH
404 radicals (measures the decrease in DPPH radical absorption after exposure to radical
405 scavengers), reducing power (measures the conversion of a Fe^{3+}/ferricyanide complex
406 to Fe^{2+}), inhibition of β-carotene bleaching (measures the capacity to neutralize the
407 linoleate-free radical and other free radicals formed in the system which attack the
408 highly unsaturated β-carotene models), and inhibition of lipid peroxidation in brain cells
409 homogenates (measures the colour intensity of MDA-TBA complex). All the samples
410 showed bioactivity (**Table 4**) and the antioxidant activity was more significant for
411 unripe fruits (lowest EC_{50} values, ranging from 5.42 to 20.83 µg/mL). Over ripened
412 fruits revealed the lowest antioxidant properties (highest EC_{50} values, ranging from
413 49.21 to 130.27 µg/mL) which are compatible to their lower phenolic and flavonoid
414 contents. Froehlicher *et al.* (2009) also related the antioxidant activity of fresh fruit,
415 dried fruit, flowering tops and flowers of *C. monogyna* from France with the contents in
416 total phenols, proanthocyanidins, and flavonoids. Nevertheless, they reported dried
417 hawthorn flower buds and flowering tops as the richest extracts, and a lower antioxidant
418 effect of fresh and dried fruits. Probably, the fruits used in that study were in a ripened
419 or over ripened stage. Plant stress response involves the synthesis of several secondary
420 metabolites of the phenylpropanoid pathway such as phenolics (Strycharz and Shetty,
421 2002). Therefore the production of these compounds is increased in unripe fruits, being

422 subsequently used as antioxidants along the maturity of the fruits, leading to a phenolics
423 decrease in the ripened and over ripened stages (**Table 4**).
424 Bernatonienė *et al.* (2008) studied the DPPH radical scavenging activity of ethanolic
425 and aqueous extracts of *C. monogyna* fruits and individual substances (chlorogenic acid,
426 hyperoside, rutin, quercetin, vitexin-*O*rhamnoside, epicatechin, catechin, and
427 procyanidin), and they reported weaker free radical-scavenging properties than a
428 combination of these substances. Herein, the antioxidant properties were evaluated
429 using the whole extract, taking advantage of the complex mixture of phytochemicals
430 with potential additive and synergistic effects (Liu, 2004). The studied flower buds,
431 flowers and unripe fruits revealed a higher DPPH scavenging activity than the hawthorn
432 berries from Serbia (52.04 µg/mL as EC_{50} value) studied by Tadić *et al.* (2008). In fact
433 flower buds, flowers and, mostly unripe fruits gave better results for DPPH radical
434 scavenging effects and reducing power than the standard trolox.
435
436 The present work shows the potential of different parts of *Crataegus monogyna* as
437 sources of several compounds, including nutrients and nutraceuticals, certainly involved
438 in their nutritional and medicinal uses. The flowers revealed the highest tocopherols and
439 ascorbic acid contents, as also the best n-6/n-3 fatty acids ratio. Over ripened fruits
440 showed the highest levels of carbohydrates, free sugars, β-carotene and SFA. Unripe
441 fruits presented the highest PUFA contents with the best PUFA/SFA ratio, as also the
442 highest levels of phenolics and the most promising antioxidant properties, even better
443 than the standard. Therefore, this plant should be considered a healthy product as it is
444 documented in local ethnobotanical uses, such as the use of flower buds and young

leaves raw in salads or prepared in pickles, corymbs infusions and decoctions, liniments of unripe and ripened fruits and liquors or marmalades made of over ripened pomes.

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Figure1. Different parts of *Crataegus monogyna* used as traditional food and folk medicine in Trás-os-Montes, Portugal. The top leaves are visible attached at the base of the corymbs (inflorescence).

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 *Crataegus monogyna* parts (mean ± SD; n=3). In each row, different letters mean significant differences ($p<0.05$).

	Flower buds	Flowers	Unripe fruits	Ripened fruits	Over ripened fruits
Moisture	70.68 ± 5.12 b	69.83 ± 4.31 c	76.81 ± 3.17 a	60.00 ± 6.10 d	59.76 ± 5.35 e
Ash	6.78 ± 0.15 b	7.25 ± 0.26 a	2.39 ± 0.18 d	3.21 ± 0.09 c	3.25 ± 0.05 c
Carbohydrates	75.71 ± 0.17 c	77.42 ± 1.30 b	91.24 ± 0.24 a	91.99 ± 0.13 a	92.75 ± 0.23 a
Proteins	15.18 ± 0.22 a	11.75 ± 1.37 b	5.57 ± 0.30 c	3.97 ± 0.09 d	3.40 ± 0.20 d
Fat	2.33 ± 0.01 b	3.57 ± 0.15 a	0.80 ± 0.05 c	0.83 ± 0.00 c	0.60± 0.00 d
Energy	384.53 ± 0.40 d	388.84 ± 1.26 c	394.43 ± 0.70 a	391.32 ± 0.26 b	389.98 ± 0.15 cb
Fructose	2.17 ± 0.03 d	3.44 ± 0.07 c	0.29± 0.01 e	7.24 ± 0.56 b	10.16 ± 0.12 a
Glucose	7.54 ± 0.12 c	7.91 ± 0.18 c	1.48 ± 0.04 d	33.39 ± 2.52 b	44.44 ± 0.91 a
Sucrose	1.19 ± 0.03 b	2.02 ± 0.05 a	0.36 ± 0.03 d	0.14 ± 0.03 e	0.66 ± 0.03 c
Trehalose	0.48 ± 0.05 a	0.11 ± 0.01 d	0.08 ± 0.01 d	0.25 ± 0.00 c	0.36 ± 0.02 b
Total Sugars	11.38 ± 0.23 c	13.48 ± 0.32 c	2.21 ± 0.06 d	41.03 ± 3.12 b	56.07 ± 1.07 a

Table 2. Composition of *Crataegus monogyna* parts in fatty acids (mean ± SD; n=3). In each column different letters mean significant differences ($p<0.05$).

	Flower buds	Flowers	Unripe fruits	Ripened fruits	Over ripened fruits
C10:0	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.00	0.15 ± 0.01	0.15 ± 0.01
C12:0	0.43 ± 0.04	0.34 ± 0.09	0.47 ± 0.04	1.91 ± 0.19	2.08 ± 0.02
C14:0	0.41 ± 0.07	0.74 ± 0.20	0.42 ± 0.04	0.85 ± 0.01	1.04 ± 0.03
C14:1	0.08 ± 0.01	0.15 ± 0.07	0.11 ± 0.01	0.12 ± 0.01	0.06 ± 0.00
C15:0	0.16 ± 0.04	0.19 ± 0.07	0.23 ± 0.05	0.21 ± 0.01	0.29 ± 0.00
C16:0	10.61 ± 0.48	11.23 ± 0.13	11.02 ± 0.15	13.73 ± 0.07	15.52 ± 0.22
C16:1	0.17 ± 0.01	0.15 ± 0.01	0.08 ± 0.01	0.24 ± 0.02	0.37 ± 0.03
C17:0	0.21 ± 0.03	0.25 ± 0.02	0.20 ± 0.01	0.34 ± 0.04	0.55 ± 0.00
C18:0	1.67 ± 0.02	2.43 ± 0.32	1.75 ± 0.05	2.43 ± 0.07	2.96 ± 0.10
C18:1n9c	2.74 ± 0.02	2.16 ± 0.26	9.82 ± 0.27	13.92 ± 0.07	7.68 ± 0.42
C18:2n6c	15.64 ± 0.39	14.17 ± 0.06	58.48 ± 0.50	17.53 ± 0.18	13.12 ± 0.64
C18:3n3	26.79 ± 0.14	29.51 ± 0.11	5.98 ± 0.44	7.41 ± 0.29	15.65 ± 0.74
C20:0	1.10 ± 0.01	1.56 ± 0.13	0.86 ± 0.04	1.63 ± 0.02	1.78 ± 0.07
C20:1c	0.17 ± 0.02	0.22 ± 0.00	0.52 ± 0.06	0.27 ± 0.01	0.20 ± 0.01
C20:2c	0.11 ± 0.01	0.14 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	nd
C20:3n3+C21:0	0.15 ± 0.01	0.20 ± 0.00	0.07 ± 0.00	nd	nd
C22:0	0.60 ± 0.04	0.87 ± 0.01	0.74 ± 0.12	2.05 ± 0.07	2.03 ± 0.17
C23:0	36.95 ± 0.02	33.67 ± 0.70	8.18 ± 0.15	32.77 ± 0.33	30.40 ± 1.87
C24:0	1.98 ± 0.06	1.98 ± 0.33	0.95 ± 0.11	4.41 ± 0.08	6.15 ± 0.08
Total SFA	54.16 ± 0.36 c	53.31 ± 0.32 c	24.87 ± 0.14 d	60.48 ± 0.58 b	62.93 ± 1.83 a
Total MUFA	3.15 ± 0.16 d	2.68 ± 0.28 d	10.52 ± 0.22 b	14.54 ± 0.11 a	8.11 ± 0.46 c
Total PUFA	42.58 ± 0.21 c	43.88 ± 0.11 b	64.53 ± 0.07 a	24.94 ± 0.47 e	28.96 ± 1.37 d
PUFA/SFA	0.79 ± 0.01 c	0.82 ± 0.01 b	2.59 ± 0.01 a	0.41 ± 0.01 d	0.46 ± 0.04 d
n-6/n-3	0.58 ± 0.02 c	0.48 ± 0.00 c	9.81 ± 0.80 a	2.37 ± 0.07 b	0.84 ± 0.00 c

nd- not detected. Capric acid (C10:0); Lauric acid (C12:0); 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 (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1c); *cis*-11,14-Eicosadienoic acid (C20:2c); *cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).

Table 3. Composition of *Crataegus monogyna* parts in tocopherols, ascorbic acid and β-carotene (mg/100 g dry weight) (mean ± SD; n=3). In each row different letters mean significant differences ($p<0.05$).

	Flower buds	Flowers	Unripe fruits	Ripened fruits	Over ripened fruits
α-tocopherol	61.64 ± 13.50 b	110.09 ± 17.53 a	16.03 ± 1.23 c	113.42 ± 15.25 a	57.34 ± 3.32 b
β-tocopherol	2.94 ± 0.43 b	4.27 ± 0.59 a	1.02 ± 0.03 c	2.33 ± 0.45 b	2.23 ± 0.06 b
γ-tocopherol	15.72 ± 1.87 b	22.76 ± 3.08 a	3.87 ± 0.13 c	3.34 ± 0.50 c	3.53 ± 0.04 c
δ-tocopherol	16.39 ± 0.84 b	22.73 ± 3.86 a	0.84 ± 0.02 c	0.90 ± 0.07 c	0.88 ± 0.03 c
Total tocopherols	96.70 ± 16.63 bc	159.84 ± 25.06 a	21.75 ± 1.41 d	119.99 ± 16.27 ba	63.99 ± 3.39 c
Ascorbic acid	141.29 ± 1.52 c	408.37 ± 2.96 a	130.33 ± 0.74 c	220.24 ± 5.04 b	28.40 ± 3.15 d
β-carotene	48.48 ± 0.05 c	52.62 ± 0.02 c	16.42 ± 0.01 d	54.84 ± 0.10 b	94.15 ± 0.11 a

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

	Flower buds	Flowers	Unripe fruits	Ripened fruits	Over ripened fruits	Trolox
η (%)	36.83 ± 3.22 c	36.67 ± 2.48 c	13.68 ± 1.20 d	83.01 ± 6.22 a	60.97 ± 4.89 b	-
Phenolics (mg GAE/g extract)	275.25 ± 24.32 c	330.32 ± 1.82 b	701.65 ± 16.57 a	274.27 ± 5.91 c	247.03 ± 9.32 c	-
Flavonoids (mg CE/g extract)	106.84 ± 0.71 b	103.78 ± 7.65 b	436.34 ± 43.36 a	21.70 ± 0.82 c	22.26 ± 0.45 c	-
DPPH scavenging activity	36.65 ± 7.34 b	30.78 ± 1.79 cb	20.83 ± 2.36 c	121.31 ± 14.10 a	130.27 ± 6.27 a	43.03 ± 1.71 b
Reducing power	18.55 ± 1.36 c	18.71 ± 0.86 c	17.42 ± 1.33 c	55.80 ± 2.78 b	75.54 ± 4.53 a	29.62 ± 3.15 c
β-carotene bleaching inhibition	98.22 ± 13.89 a	52.33 ± 7.65 b	6.38 ± 0.06 c	80.50 ± 1.93 a	100.49 ± 14.98 a	2.63 ± 0.14 c
TBARS inhibition	35.96 ± 6.05 a	12.68 ± 1.47 cb	5.42 ± 0.37 c	32.90 ± 1.22 a	49.21 ± 2.08 a	3.73 ± 1.90 d