Strawberry-tree, blackthorn and rose fruits: detailed characterization in nutrients and phytochemicals with antioxidant properties

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Abstract

The chemical composition and biological properties of three wild fruits (strawberry-tree berries, sloes and dog rose hips) were evaluated, in order to valorise these products as sources of nutrients and nutraceuticals. The analysed fruits contain very useful bioactive phytochemicals such as phenolics, vitamins (ascorbic acid and tocopherols) and carotenoids. All the samples proved to have antioxidant activity (measured by four different in vitro assays) being more significant for rose fruits (EC_{50} values lower than 90 μg/mL). The combination of bioactive compounds and rich nutritional composition (high contents in carbohydrates, low contents in fat with the precious contribution of polyunsaturated fatty acids, precursors of omega-3 and omega-6 fatty acids) of the studied wild fruits make them a very special food.

Keywords: Wild fruits; nutritional value; phytochemicals; bioactive properties

1. Introduction

The fruits of *Arbutus unedo* L. (Castroviejo, 1996) (Ericaceae), a species widely distributed in the Mediterranean region and North Africa, are a red aggregate drupe generally known as strawberry-tree berries. Their use is very popular in Portugal, particularly made in a kind of strong brandy, the 'aguardente de medronho'. In several Portuguese regions (Trás-os-Montes, Alentejo and Algarve) the fruits eaten raw or made in liqueurs, as well as, bark or roots decoctions, are used as anti-inflammatory, laxative, carminative, digestive, odontalgic and cardiotonic (Novais, Santos, Mendes, & Pinto-Gomes, 2004; Salgueiro, 2004; Carvalho, 2005; Camejo-Rodrigues, 2006). Some literature report the traditional use of the leaves as a diuretic, urinary antiseptic, antidiarrheal, astringent, depurative, against blenorrhagia, diabetes and as antihypertensive (Ziyyat et al., 1997). Experimental investigations have already shown that the aqueous extract of the plant exhibited antihypertensive (Ziyyat & Boussairi, 1998) and vasorelaxant (Ziyyat et al., 2002) activities. Sloes, the fruits of blackthorn, *Prunus spinosa* L. (Castroviejo, 1998), a deciduous shrub native to Europe, have also been used as astringent, diuretic and purgative (Lust, 1980). In the North-eastern Portugal, the fruits are commonly eaten raw, prepared in jams or macerated with sugar, honey and brandy to obtain a digestive and laxative liqueur, which is usually drunk after copious meals (Novais et al., 2004; Salgueiro, 2004; Carvalho, 2005; Camejo-Rodrigues, 2006). Moreover, rose hips, i.e. the pomaceous fruit of dog roses, *Rosa canina* L., possess prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis (Rein, Kharazmi, & Winther, 2004; Kharazmi, 2008), rheumatism, gout, and sciatica, for diseases with fever, for colds and infectious diseases including influenza, against gastrointestinal disorders, to

54 aid digestion, prevention of inflammation of the gastric mucosa and gastric ulcer, for
55 gallstones, biliary complaints, as a laxative, for disorders of the kidney and the lower
56 urinary tract, as a diuretic, for dropsy and as an astringent (Orhan, Hartevioğlu, Küpeli,
57 & Yesilalada, 2007). In Portugal, rose hips from the Caninae DC. section (Castroviejo,
58 1998), a polymorphic group of scrambling rose species indigenous to Europe, northwest
59 Africa and western Asia, are also used in the treatment of colds, influenza, minor
60 infectious diseases, diarrhoea and as topical anti-inflammatory for muscular-skeletical
61 pathologies. Immature and ripened fruits of wild roses, mainly *Rosa canina* L and *Rosa*
62 *corymbifera* Borkh. (Castroviejo, 1998), were/are indistinctively applied in the folk
63 medicine of the Trás-os-Montes region. During late summer, the rose hips were
64 gathered and eaten raw as snacks or given raw to the children, who were taught how to
65 eat the outer fleshy hypantium and to spit out the dry single-seeded fruits (achenes) that
66 are embedded in a matrix of fine, but stiff, hairs (frequency of citation > 40%)
67 (Carvalho, 2005). In the surveyed area (north-eastern Portuguese region) wild fruits
68 were, sometime still are, commonly preserved and stored, for consumption during the
69 long and hard winters. Jams are prepared from arbutus berries, sloes, blackberries
70 (*Rubus ulmifolius* Schott.) and wild strawberries (*Fragaria vesca* L.). Despite the
71 brandy of arbutus berries, quite exceptional and much appreciated spirits are made from
72 sloes, blackberries and wild strawberries (frequency of citation > 55%) (Carvalho,
73 2005).
74 Epidemiological studies have consistently shown an inverse association between
75 consumption of vegetables and fruits and the risk of certain forms of cancer and
76 cardiovascular diseases (Bazzano, He, & Ogden, 2001). The protective effects have
77 been primarily attributed to antioxidants, such as Vitamin C, Vitamin E, β-carotene and,

lately, to phenolic compounds (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). There are only a few reports on antioxidant composition of strawberry-tree (Spanish samples; Pallalauf, Rivas-Gonzalo, Castillo, Cano, & Pascual-Teresa, 2008) and rose fruits (German samples; Wenzig et al., 2008). Considering the nutritional composition, the studies available in literature only report macronutrient analysis (Demir & Özcan, 2001; Marakoğlu, Arslan, Özcan, & Hacıseferoğulları., 2005; Özcan & Hacıseferoğulları, 2007; Pallalauf et al., 2008).

Despite the high popularity of these wild fruits in Portugal, data regarding a complete nutritional and phytochemical characterization are missing. The high nutritional quality and bioactive compounds of these fruits are likely to be lost if not documented. Herein, we intend to present a study of the chemical composition and antioxidant properties of three Portuguese wild fruits (strawberry-tree, sloes and rose hips), in order to valorise these products as sources of nutrients and nutraceuticals. Chemical analysis included determination of proteins, fats, ash, and carbohydrates, and individual profiles in sugars and fatty acids by chromatographic techniques. Phytochemicals such as phenolics, flavonoids, vitamins (tocopherols and ascorbic acid), and carotenoids were also determined.

2. Materials and methods

2.1. Samples

The fruits of *Arbutus unedo*, *Prunus spinosa* and *Rosa canina* sl. were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, North-eastern Portugal, according to the folk uses of each species (**Table 1**), especially those concerning fruit

ripening stage and the most suitable gathering period and practices. Strawberry-tree berries were collected fully ripened in November 2008; well matured sloes and rose hips were gathered in late September 2008. Morphological key characters from the Flora Iberica (Castroviejo, 1996; Castroviejo, 1998) were used for plant identification. The fruits with seeds 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). All the other solvents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan (Lisbon, Portugal), while toluene and sulphuric acid were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, tocopherol standards (α, β, γ and δ), and the standards used in the antioxidant activity assays: 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 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Nutrients composition

125 *Macronutrients.* The samples were analysed for chemical composition (moisture,
126 protein, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The
127 crude protein content (N × 6.25) of the samples was estimated by the macro-Kjeldahl
128 method; the crude fat was determined by extracting a known weight of powdered
129 sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined
130 by incineration at 600±15 ºC. Total carbohydrates were calculated by difference: Total
131 carbohydrates = 100 – (g moisture + g protein + g fat + g ash). Total energy was
132 calculated according to the following equations: Energy (kcal) = 4 × (g protein +g
133 carbohydrate) + 9 × (g lipid).
134
135 *Fatty Acids.* Fatty acids were determined by gas-liquid chromatography with flame
136 ionization detection (GC-FID)/capillary column as described previously by the authors
137 (Barros, Venturini, Baptista, Estevinho, & Ferreira, 2008), and after the following trans-
138 esterification procedure: fatty acids (obtained after Soxhlet extraction) were methylated
139 with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v), during at least 12 h in a bath
140 at 50 ºC and 160 rpm; then 3 mL of deionised water were added, to obtain phase
141 separation; the FAME were recovered with 3 mL of diethyl ether by shaking in vortex ,
142 and the upper phase was passed through a micro-column of sodium sulphate anhydrous,
143 in order to eliminate the water; the sample was recovered in a vial with Teflon, and
144 before injection the sample was filtered with 0.2 µm nylon filter from Milipore. The
145 fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with
146 a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel
147 column (30 m x 0.32 mm ID x 0.25 µm d_f). The oven temperature program was as
148 follows: the initial temperature of the column was 50 ºC, held for 2 min, then a

149 10ºC/min ramp to 240 ºC and held for 11 min. The carrier gas (hydrogen) flow-rate was
150 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250
151 ºC. For each analysis 1 µL of the sample was injected in GC. Fatty acid identification
152 was made by comparing the relative retention times of FAME peaks from samples with
153 standards. The results were recorded and processed using CSW 1.7 software (DataApex
154 1.7) and expressed in relative percentage of each fatty acid.
155
156 *Sugars.* Free sugars were determined by high performance liquid chromatography
157 coupled to a refraction index detector (HPLC-RI) as described by Barros et al. (2008)
158 with some modifications. Dried sample powder (1.0 g) was spiked with the melezitose
159 as internal standard (IS, 5 mg/ml), and was extracted with 40 mL of 80% aqueous
160 ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged (Centorion
161 K24OR- 2003 refrigerated centrifuge) at 15,000 g for 10 min. The supernatant was
162 concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of
163 ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved
164 in water to a final volume of 5 mL. Soluble sugars were determined by using HPLC
165 (Knauer, Smartline system) at 35 ºC. The HPLC system was equipped with a Knauer
166 Smartline 2300 RI detector and with a Eurospher 100-5 NH_2 column (4.6 x 250 mm, 5
167 mm, Knauer). The mobile phase was acetonitrile/deionized water, 7:3 (v/v) at a flow
168 rate of 1 mL/min. The results are expressed in g/100 g of dry weight, calculated by
169 internal normalization of the chromatographic peak area. Sugar identification was made
170 by comparing the relative retention times of sample peaks with standards. The sugar
171 standards used for identification were purchased from Sigma Chemical Co. (St. Louis,
172 USA): L(+)-arabinose, D(-)-fructose, L-fucose, D(+)-galactose, D(+)-glucose

anhydrous, lactose 1-hydrate, maltose 1-hydrate, maltulose monohydrate, D(+)-mannitol, D(+)-mannose, D(+)-melezitose, D(+)-melibiose monohydrate, D(+)-raffinose pentahydrate, L(+)-rhamnose monohydrate, D(+)-sucrose, D(+)-trehalose, D(+)- turanose and D(+)-xylose.

2.4. Phytochemicals composition

Tocopherols. Tocopherols content was determined following a procedure previously optimized and described by Barros, Heleno, Carvalho, & Ferreira (in press). BHT solution in hexane (10 mg/mL; 100 µL) and IS solution in hexane (tocol; 50 µg/mL; 400 µL) were added to the sample prior to the extraction procedure. The samples (~500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000g) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated with anhydrous sodium sulphate, filtered through a 0.22 µm disposable LC filter disk, transferred into a dark injection vial and analysed by HPLC. The HPLC equipment consisted of an integrated system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer, Germany) connected in series with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250 x 4.6 mm) normal-phase column from YMC Waters (Japan)

operating at 30ºC (7971 R Grace oven). The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherol contents in the samples are expressed in µg per g of dry sample.

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; $y = 3.0062x + 0.007$; $R^2 = 0.9999$), and the results were expressed as µg of ascorbic acid/g of dry weight.

Carotenoids. β-Carotene and lycopene 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. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 mL) = $- 0.0458 \times A_{663} + 0.204 \times A_{645} + 0.372 \times A_{505} -$

220 0.0806 × A_{453}; β-carotene (mg/100 mL) = 0.216 × A_{663} – 1.220 × A_{645} - 0.304 × A_{505} +
221 0.452 × A_{453}. The results were expressed as μg of carotenoid/g of dry weight.
222
223 *Phenolics.* A fine dried powder (20 mesh; ~1g) was extracted by stirring with 50 mL of
224 methanol at 25 ºC at 150 rpm for 12 h and filtered through Whatman No. 4 paper. The
225 residue was then extracted with one additional 50 mL portion of methanol. The
226 combined methanolic extracts were evaporated at 35ºC under reduced pressure (rotary
227 evaporator Büchi R-210), re-dissolved in methanol at a concentration of 10 mg/mL, and
228 stored at 4 ºC for further use.
229 Total phenolics were estimated based on procedures described by Wolfe, Wu, & Liu
230 (2003) with some modifications. An aliquot of the extract solution (1 mL) was mixed
231 with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10 v/v) and sodium
232 carbonate (75 g/L, 4 mL). The tubes were vortexed for 15 s and allowed to stand for
233 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm.
234 Gallic acid was used to calculate the standard curve (0.05-0.8 mM; $y = 1.9799x$ +
235 0.0299; $R^2 = 0.9997$), and the results were expressed as mg of gallic acid equivalents
236 (GAEs) per g of extract.
237 Total flavonoid content was determined using the method of Jia Tang, & Wu (1999),
238 with some modifications. An aliquot (0.5 mL) of the extract solution was mixed with
239 distilled water (2 mL) and subsequently with $NaNO_2$ solution (5%, 0.15 mL). After 6
240 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand further 6 min,
241 thereafter, NaOH solution (4%, 2 mL) was added to the mixture. Immediately, distilled
242 water was added to bring the final volume to 5 mL. Then the mixture was properly
243 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; $y = 0.9186x - 0.0003$; $R^2 = 0.9999$) and the results were expressed as mg of (+)-chatequin equivalents (CEs) per g of extract.

2.5. In vitro evaluation of the antioxidant properties

Chemical assays already described by the authors in previous studies (Barros et al., in press), were applied to evaluate the antioxidant activity of all the samples. Different concentration of the extracts (10 mg/mL to 0.05 mg/mL) were used to find EC_{50} values.

DPPH radical-scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one 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 ($6x10^{-5}$ mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

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.

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. 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

292 homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a
293 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An
294 aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of
295 the extracts (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 ml) and ascorbic acid (0.1
296 mM; 0.1 ml) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic
297 acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and
298 the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10
299 min to remove the precipitated protein, the colour intensity of the malondialdehyde
300 (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm.
301 The inhibition ratio (%) was calculated using the following formula: Inhibition ratio
302 (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the
303 compound solution, respectively. The extract concentration providing 50% lipid
304 peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition
305 percentage against extract concentration. Trolox was used as standard.
306
307 *2.6. Statistical analysis*
308 For each one of the species three samples were analysed and also all the assays were
309 carried out in triplicate. The results are expressed as mean values and standard deviation
310 (SD) or standard errors (SE). The results were analyzed using one-way analysis of
311 variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was
312 carried out using SPSS v. 16.0 program.
313

3. Results and discussion

We performed a nutritional and pytochemical characterization of three wild Portuguese fruits: Strawberry-tree (*A. unedo*), blackthorn (*P. spinosa*) and rose (*R. canina* sl.) fruits. The ethnobotanical survey conducted in the North-eastern Portugal (Carvalho, 2005) has reported that in former times the studied wild fruits were often eaten as snacks during the working day in the crop fields, in the meadows while the cattle was grazing or in the forest. Moreover the mothers forced their children to eat them raw when fully ripened because they were convinced it was healthy. As several studies have also documented (González-Tejero et al., 2008; Hadjichambis et al., 2008) the wild fruits resulting products such as marmalades, spirits and infusions were also deliberately consumed for their preventative or curative properties and are considered 'medicinal foods'(Carvalho, 2005; González-Tejero et al., 2008).

3.1. Nutrients composition

The results of the nutrients composition and estimated energetic value (expressed on dry weight basis) obtained for the wild fruits are shown in **Table 1**. Blackthorn fruits revealed the highest moisture content (60.86 g/100 g), a similar value to the described by Marakoğlu et al. (2005) for Turkish samples (69.37%). Portuguese strawberry-tree fruits revealed a slightly higher moisture content (59.70 g/100 g) than Turkish fruits (53.72%) (Özcan & Hacıseferoğulları, 2007). Otherwise, Portuguese rose fruits showed lowest moisture contents (48.68 g/100 g) than Turkish samples (69.52%) (Hacıseferoğulları, Özcan, Sonmete, & Özbek, 2005).

Carbohydrates, calculated by difference, were the most abundant macronutrients and were higher than 88.5%. In fact, these fruits are rich in different carbohydrates, either

338 monosaccharides or polysaccharides such as cellulose and starch (Demir & Özcan,
339 2001; Özcan & Hacıseferoğulları, 2007).
340 The results show that the consumption of these fruits as snacks was appropriate for the
341 particular purpose of satisfying hunger in view of their carbohydrates content. In fact
342 some informants highlighted the importance of wild plants in local daily diet
343 specifically during famine periods, such as those occurring when the Spanish Civil War
344 and the Second World War (Carvalho, 2005).
345 Protein was found in low levels and varied between 2.72 g/100 g in rose fruits and 3.09
346 g/100 g in strawberry-tree fruits. The Turkish samples of rose (6.71 to 8.44%, Demir &
347 Özcan, 2001), blackthorn (3.4%, Marakoğlu et al., 2005) and strawberry-tree (3.36%,
348 Özcan & Hacıseferoğulları, 2007) showed highest proteins levels. Fat was the
349 macronutrient less abundant being lower than 2% and also lower than those found in
350 Turkish fruits: 2.1% (Özcan & Hacıseferoğulları, 2007), 2.06% (Marakoğlu et al., 2005)
351 and 1.2 to 1.6% (Demir & Özcan, 2001) for strawberry-tree, blackthorn and rose,
352 respectively. On the basis of the proximate analysis, it can be calculated that a dry
353 portion of 100 g of these fruits assures, on average, 394 Kcal. The highest values are
354 guaranteed by strawberry-tree fruits, while blackthorn fruits give the lowest energy
355 contribution (**Table 1**). Ash content fall between proteins and fat contents, being more
356 abundant in blackthorn fruits (6.65 g/100 g), a much higher value than the one found in
357 Turkish samples (2.72%, Marakoğlu et al., 2005). The lowest values were found in
358 strawberry-tree fruits (1.71 g/100 g), which was lower than the value reported in the
359 Turkish samples (2.82%, Özcan & Hacıseferoğulları, 2007).
360 Despite some similarities in the composition of Portuguese and Turkish samples, it is
361 known that differences in chemical properties of fruits having about the same size were

362 probably due to environmental conditions in conjunction with the analytical methods
363 used. In addition, moisture, crude protein, fibre, fat and ash contents of fruits are
364 affected chiefly by variety and growth conditions (Hacıseferoğulları et al., 2005).
365
366 The results for fatty acid composition, total saturated fatty acids (SFA),
367 monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the
368 studied wild fruits are shown in **Table 2**.The major fatty acids found in strawberry-tree
369 and rose fruits were α-linolenic acid (C18:3) and linoleic acid (C18:2), contributing to
370 the prevalence of PUFA in these samples. α-Linolenic and linoleic acids are essential
371 fatty acids as they cannot be synthesised by the human organism, due to the lack of
372 desaturase enzymes required for their production. They must be obtained by the diet and
373 originate the omega-3 and omega-6 fatty acids, respectively (Voet & Voet, 2004). These
374 omega fatty acids are the biosynthetic precursor of eicosanoids, meaning that their
375 intake concentrations will strongly influence eicosanoids production, and, therefore, the
376 organism's metabolic functions. Furthermore, they can also decrease the total amount of
377 fat in blood (cholesterol), reducing the risk of cardiovascular diseases (Voet & Voet,
378 2004; Kanu et al., 2007). In blackthorn fruits, PUFA predominated over MUFA due to
379 the abundance of oleic acid (C18:1). These fruits also present high levels of linoleic acid
380 but significant lower amounts of α-linolenic acid than the other two wild fruits. In all
381 the cases UFA predominate over SFA, ranging from 80 to 84%, being palmitic acid the
382 main SFA found. Twenty four fatty acids were identified and quantified. As far as we
383 know, nothing has been reported on fatty acid composition of strawberry-tree and
384 blackthorn fruits. Nevertheless, Wenzig et al. (2008) also reported palmitic, linoleic and

385 α-linolenic acid as the main free fatty acids present in rose hip extracts from German
386 fruits.
387
388 In what concerns sugar composition (**Table 3**) the wild fruits presented fructose,
389 glucose and sucrose as main sugars. The present study describes for the first time the
390 sugars composition in these wild fruits. For strawberry-tree (24.21 g/100 g) and rose
391 fruits (12.89 g/100 g) fructose was the most abundant sugar, while glucose
392 predominates in blackthorn samples (29.84 g/100 g). Strawberry-tree fruits revealed the
393 highest total sugars content, and highest levels of fructose and sucrose, which is in
394 agreement with its sweet taste. Otherwise, rose fruits showed the lowest levels in total
395 sugars (26.90 g/100 g).
396 Sugars contents (**Table 3**) are a significant part of carbohydrates (**Table 1**), but other
397 carbohydrates are also present in these fruits such as polysaccharides (eg. cellulose and
398 starch) (Demir & Özcan, 2001; Özcan & Hacıseferoğulları, 2007).
399
400 Overall, strawberry-tree fruits revealed the highest energetic value, but with the highest
401 carbohydrates and proteins content. Concerning sugar composition it presents the
402 highest percentage of fructose, which is one of the important dietary monosaccharides
403 and it is known to be the sweetest of all naturally occurring carbohydrates (Hanover &
404 White, 1993). Strawberry-tree (*A. unedo*) fruits are considered an excellent edible to
405 make delicious and nourishing jams and conserves due to its sweetness and delicate
406 pleasant flavour. Rose fruits presented the highest PUFA, which may be relevant since
407 α-linolenic and linoleic acids are precursors of omega-3 and omega-6 fatty acids often
408 related to an increase in HDL cholesterol and decrease in LDL cholesterol,

triacylglycerol, lipid oxidation, and LDL susceptibility to oxidation (Voet & Voet, 2004; Kanu et al., 2007). Although the edible use of rose fruits is less frequent in Portugal, than arbutus berries and sloes, even than its medicinal use, rose hips are considered an excellent ingredient for making delicious jams, syrups and herbal teas, in several European regions (Tardío, Pardo de Santayana, & Morales, 2006; González-Tejero et al., 2008; Hadjichambis et al., 2008).

3.2. Phytochemicals composition

Vitamins (tocopherols and ascorbic acid) and carotenoids contents in the wild fruits were determined and the results are given in **Table 4**.

The values obtained in the analysis of the samples point to the existence of differences in what concerns tocopherols composition. α-Tocopherol was the major compound in all the fruits, and δ-tocopherol was only detected in blackthorn fruits. Strawberry-tree fruits presented the highest content of tocopherols (23.46 mg/ 100 g of dry weight; **Table 4**, **Figure 1**) while rose fruits revealed the lowest content (8.33 mg/100 g). Even if considering the fact that fruits in general have very low content of this vitamin, characteristic of fat-rich foods, the content was not negligible. Some authors published tocopherols determination in Spanish strawberry-tree fruits, but reporting only the presence of α-tocopherol (Pallalauf et al., 2008). In our study, we used a different extraction methodology with the introduction of an antioxidant protector to minimize tocopherols loss, and we used fluorescence detection, which is more sensitive than UV detection, allowing us to quantify other vitamers such as α, β, and γ-tocopherols. Regarding tocopherols in rose and blackthorn fruits, as far as we know, nothing is described in literature. The health benefits of tocopherol as a bioactive compound are

433 well documented. α-Tocopherol, the principal form of vitamin E, is a lipid-soluble
434 antioxidant and it functions as a chain-breaking antioxidant for lipid peroxidation (LP)
435 in cell membranes and also as a scavenger of ROS (Reactive Oxygen Species) such as
436 singlet oxygen. It is considered to serve as the first line of defence against LP, and it
437 protects PUFAs (polyunsaturated fatty acids) in cell membranes from free radical attack
438 through its scavenging activity in biomembranes at early stages of LP (Kanu et al.,
439 2007).
440 Ascorbic acid was the most abundant vitamin in blackthorn and rose fruits, and
441 particularly for the latter sample it presented a very high level (68.04 mg/100 g dry
442 weight; **Table 4**). In fact, rose fruits have been used as a source of vitamin C in tea and
443 other products for many years (Krharazmi, 2008). Weinzig et al. (2008) reported the
444 quantification of ascorbic acid in German rose fruits, but the values are expressed in %
445 of dry extract and not of dry sample, which can not be comparable. Also, the
446 quantification of vitamin C in Spanish strawberry-tree fruits was reported (Pallalauf et
447 al., 2008), being the values described lower than the contents found in our Portuguese
448 fruits. Levels of vitamin C could be comparable to those in fruits like peaches, apples or
449 plums. Regarding blackthorn fruits, as far as we know, nothing is described in literature.
450 Carotenoids are widespread pigments in plants, being involved in photosynthesis and
451 photoprotection (Hodisan, Socaciu, Ropan, & Neamtu, 1997); β-carotene was found in
452 small amounts in all the fruits (lower than 1.3 mg/100 g dry weight) and lycopene was
453 only detected in rose fruits. This is in agreement with the results of rose fruits from
454 Romania reported by Hodisan et al. (1997). The antioxidant properties of carotenoids
455 have been suggested as being the main responsible for their beneficial effects (Rao &
456 Rao, 2007). Particularly, β-carotene has been found to be inversely associated with

457 cancer risk in epidemiologic studies and showed promising results in laboratory assays.
458 Also, the role of lycopene in the prevention of chronic diseases has been evaluated in
459 epidemiological studies as well as in tissue culture experiments using human cancer cell
460 lines, animal studies and also human clinical trials (Rao & Rao, 2007).
461
462 **Table 5** presents the yields of the methanolic extraction and the phenolics and
463 flavonoids concentrations obtained in the wild fruits extracts. It was not observed any
464 correlation between the extraction yield and the phenolics contents. Phenolics were the
465 major antioxidant components (83.40-143.17 mg/g of extract) and rose fruits revealed
466 the highest content in phenolics. The amount found in our sample was higher than the
467 ones found in German rose fruits (82.2 to 133.1 mg/g of extract) (Wenzig et al., 2008).
468 Phenolic compounds exhibit a wide range of biological effects including antibacterial,
469 anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral,
470 anticarcinogenic and vasodilatory actions (Soobrattee et al., 2005); many of these
471 biological functions have been attributed to their free radical scavenging and antioxidant
472 activity. Flavonoids are the most common and widely distributed group of plant
473 phenolics, and have been shown to be highly effective scavengers of most types of
474 oxidizing molecules, including singlet oxygen and various free radicals, which are
475 possibly involved in DNA damage and tumour promotion (Marchand, 2002). The
476 flavonoids content ranged from 8.68 to 34.99 mg/g of extract.
477
478 *3.3. In vitro evaluation of the antioxidant properties*
479 The antioxidant properties were evaluated using the whole extract, taking advantage of
480 the complex mixture of phytochemicals with potential additive and synergistic effects

481 (Liu, 2004). Several in vitro chemical and biochemical assays using animal cells were
482 performed: reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to
483 the ferrous form), scavenging activity on DPPH radicals (measuring the decrease in
484 DPPH radical absorption after exposure to radical scavengers), inhibition of β-carotene
485 bleaching (by neutralizing the linoleate-free radical and other free radicals formed in the
486 system which attack the highly unsaturated β-carotene models), and inhibition of lipid
487 peroxidation in brain tissue (measured by the colour intensity of MDA-TBA complex).
488 All the samples proved to have antioxidant activity (**table 5**) being more significant for
489 rose fruits (lowest EC_{50} values). Blackthorn fruits presented the lowest antioxidant
490 properties (highest EC_{50} values) which are compatible to its lower phenolics and
491 flavonoids content.
492
493 Overall, strawberry-trees revealed the highest contents in carbohydrates, proteins,
494 sugars, tocopherols and flavonoids, while rose fruits showed the highest content in
495 PUFA, ascorbic acid, carotenoids and phenolics, and the highest antioxidant properties.
496 The combination of the useful phytochemicals found in the analysed wild fruits and
497 their nutritional composition (particularly high contents in carbohydrates and low
498 contents in fat with the precious contribution of polyunsaturated fatty acids precursor of
499 omega-3 and omega-6 fatty acids) make them very special. This study contributes not
500 only to a better knowledge of these wild fruits but also to their valorisation.
501 The contribution of wild food plants to the total dietary intake has not yet been
502 estimated, but it is generally considered as being not very significant. However, in the
503 past, the ingestion of these fruits did provide important minor nutrients (such as
504 vitamins and essential fatty acids that were not present in daily meals mostly based on

bread and potatoes (Carvalho, 2005). Furthermore, the report of the radical scavenging activity and lipid peroxidation inhibition capacity of these species from North-eastern Portugal could help in the explanation of their uses in folk medicine against several chronic diseases known to be related to the production of ROS and oxidative stress.

Acknowledgement

The authors are grateful to the Foundation for Science and Technology (Portugal) for financial support to the research centre CIMO and L. Barros grant (SFRH/BPD/4609/2008).

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619

620 **Table 1.** Moisture (g/100 g of fresh weight), nutrients composition (g/100 g of dry
621 weight) and energetic value (Kcal/100 g of dry weight) of the wild fruits (mean ± SD;
622 n=3). In each row, different letters mean significant differences (p<0.05).
623

	Strawberry-Tree	Blackthorn	Rose
Moisture	59.70 ± 2.67 b	60.86 ± 1.69 a	48.68 ± 0.91 c
Carbohydrates	93.83 ± 0.41 a	88.51 ± 2.24 b	93.16 ± 0.18 a
Proteins	3.09 ± 0.08 a	2.86 ± 0.03 b	2.72 ± 0.05 c
Fat	1.37 ± 0.40 b	1.98 ± 0.32 a	0.65 ± 0.04 c
Ash	1.71 ± 0.09 b	6.65 ± 2.03 a	3.47 ± 0.20 b
Energy	399.99 ± 1.17 a	383.27 ± 7.09 b	398.37 ± 0.92 b

624 **Table 2.** Fatty acids composition of the wild fruits. The results are expressed as mean ±
625 SD (n=3). In each column different letters mean significant differences (p<0.05).
626

	Strawberry-Tree	Blackthorn	Rose
C6:0	0.04 ± 0.00	nd	0.05 ± 0.00
C8:0	0.04 ± 0.00	0.01 ± 0.00	0.05 ± 0.00
C10:0	0.04 ± 0.00	0.02 ± 0.00	0.09 ± 0.00
C12:0	0.65 ± 0.05	0.10 ± 0.00	0.58 ± 0.02
C13:0	0.07 ± 0.00	nd	0.07 ± 0.00
C14:0	1.34 ± 0.15	0.09 ± 0.00	0.36 ± 0.02
C14:1	nd	0.03 ± 0.00	0.02 ± 0.00
C15:0	0.10 ± 0.00	0.03 ± 0.00	0.11 ±0.01
C16:0	8.20 ± 0.25	6.50 ± 0.29	6.72 ± 0.03
C16:1	0.11 ± 0.01	0.67 ± 0.04	1.33 ± 0.05
C17:0	0.30 ± 0.01	0.11 ± 0.01	0.25 ± 0.01
C17:1c	nd	0.10 ± 0.00	nd
C18:0	4.00 ± 0.17	2.51 ± 0.15	2.41 ± 0.13
C18:1n9c	21.01 ± 0.04	57.58 ± 0.39	14.43 ± 0.28
C18:2n6c	21.50 ± 0.06	23.57 ± 0.37	39.51 ± 0.69
C18:3n3	36.51 ± 0.64	2.79 ± 0.20	26.33 ± 0.11
C20:0	0.61 ± 0.05	0.56 ± 0.04	1.00 ± 0.04
C20:1c	0.27 ± 0.02	0.06 ± 0.00	0.38 ± 0.03
C20:2c	0.16 ± 0.01	nd	0.40 ± 0.04
C20:3n3+C21:0	0.12 ± 0.01	0.03 ± 0.00	0.12 ± 0.01
C22:0	0.81 ± 0.03	0.32 ± 0.06	0.66 ± 0.06
C23:0	2.68 ± 0.10	4.42 ± 0.02	1.75 ± 0.04
C22:6n3	nd	nd	2.07 ± 0.01
C24:0	1.45 ± 0.17	0.48 ± 0.02	1.31 ± 0.03
Total SFA	20.32 ± 0.57 a	15.16 ± 0.16 b	15.40 ± 0.42 b
Total MUFA	21.39 ± 0.03 b	58.45 ± 0.34 a	16.16 ± 0.02 c
Total PUFA	58.28 ± 0.54 b	26.40 ± 0.17 c	68.44 ± 0.44 a

627 nd- not detected

Table 3. Sugars composition (g/100 g of dry weight) of the wild fruits (mean ± SD; n=3). In each row, different letters mean significant differences ($p<0.05$).

	Strawberry-Tree	Blackthorn	Rose
Fructose	24.21 ± 1.46 a	6.95 ± 0.46 c	12.89 ± 0.94 b
Glucose	12.14 ± 0.26 b	29.84 ± 1.49 a	12.17 ± 0.95 b
Sucrose	4.20 ± 0.04 a	0.27 ± 0.03 c	1.83 ± 0.27 b
Total sugars	40.55 ± 1.62 a	37.06 ± 1.92 a	26.90 ± 2.16 b

Table 4. Tocopherols, ascorbic acid and carotenoids composition (mg/100 g dry weight) of the wild fruits. The results are expressed as mean ± SD (n=3). In each row different letters mean significant differences ($p<0.05$).

	Strawberry-Tree	Blackthorn	Rose
α-tocopherol	21.98 ± 0.18 a	7.18 ± 0.34 b	7.05 ± 0.28 b
β-tocopherol	0.44 ± 0.02 a	0.06 ± 0.01 c	0.19 ± 0.01 b
γ-tocopherol	1.03 ± 0.06 b	1.91 ± 0.28 a	1.09 ± 0.06 b
δ-tocopherol	nd	0.10 ± 0.01	nd
Total tocopherols	23.46 ± 0.26 a	9.25 ± 0.64 b	8.33 ± 0.34 b
Ascorbic acid	15.07 ± 0.77 b	15.69 ± 0.53 b	68.04 ± 1.11 a
β-carotene	1.07 ± 0.09 b	0.78 ± 0.01 c	1.29 ± 0.26 a
Lycopene	nd	nd	0.51 ± 0.08

nd- not detected.

663 **Table 5.** Extraction yields, phenolics, flavonoids and antioxidant activity EC_{50} values of the wild fruits. The results are expressed as mean ± SD
664 (n=3). In each column different letters mean significant differences ($p<0.05$).
665

Antioxidant properties (EC_{50} values; µg/ml)			
DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS inhibition
447.92 ± 0.81 b	410.80 ± 0.93 b	774.99 ± 0.86 b	94.27 ± 1.21 b
597.50 ± 0.43 a	607.44 ± 0.38 a	986.90 ± 0.91 a	153.86 ± 1.98 a
428.84 ± 0.71 b	171.23 ± 0.79 c	396.06 ± 3.84 c	87.20 ± 2.17 c

666

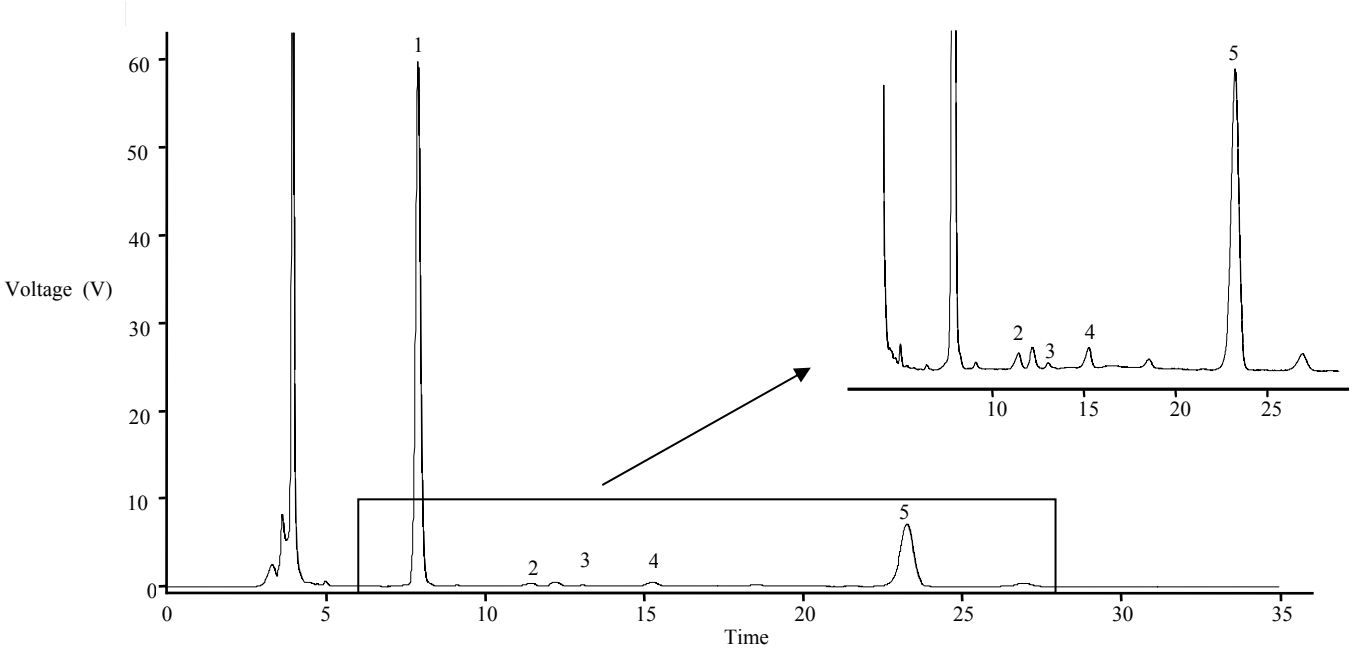

Figure 1. HPLC fluorescence chromatogram of *Arbutus unedo* (strawberry-tree) fruits. Peaks: 1- α-tocopherol; 2- BHT; 3- β-tocopherol; 4- γ-tocopherol; 5-IS (tocol).