Wild edible fruits as a potential source of phytochemicals with capacity to inhibit lipid peroxidation

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Running title: Wild fruits with capacity to inhibit lipid peroxidation.

Abstract

The edible fruits of four wild small trees or shrubs (*Arbutus unedo, Crataegus monogyna, Prunus spinosa* and *Rubus ulmifolius*) traditionally consumed in the Iberian Peninsula were studied to evaluate their potential for human nutrition, considering their content in bioactive compounds. Lipophilic phytochemicals, such as fatty acids and tocopherols, as well as some hydrophilic antioxidants, such as vitamin C (ascorbic and dehydroascorbic acids), and organic acids, were analysed. In addition, the antioxidant activity, measured as lipid peroxidation inhibition (β-carotene/linoleate and TBARS assays), was evaluated in each fruit. As far as we know, this is the first report relating to bioactive compounds in wild fruits with relation to the lipid peroxidation inhibition. Data revealed that these wild edible fruits are good sources of bioactive compounds such as ascorbic acid, tocopherols and polyunsaturated fatty acids. They could be considered as functional foods or potential sources of lipidic bioactive compounds to be included as antioxidant food ingredients or in dietary supplements, mainly *Rubus ulmifolius,* due to its high content in tocopherols. This study provides useful and relevant information that justify tocopherols influence in the prevention of lipid peroxidation, due to the strong correlation observed ($r > 0.95$) between these lipophilic bioactive compounds and the antioxidant activity.

Keywords: Wild edible fruits, Bioactive lipophilic compounds, Antioxidants, Lipid peroxidation inhibition.

1 Introduction

Lipid peroxidation has been the subject of extensive studies for several decades, and its mechanisms, dynamics and products are now quite well established. The free radical-mediated peroxidation of lipids has received a great deal of attention in connection with oxidative stress *in vivo*, as it is the case of the atherosclerosis oxidation hypothesis. The oxidative modification of low-density lipoproteins (LDL) is involved in aging and in various diseases, including atherosclerosis, cataract, rheumatoid arthritis and neurodegenerative disorders. It has a special influence in cardiovascular diseases (CVD), in which polyunsaturated fatty acids (PUFA) are particularly susceptible to oxidation mediated by free radicals. Similarly in cholesterol, PUFA auto-oxidation is initiated by a free radical hydrogen abstraction that form lipid radical followed by a rearrangement of the structure of double bond to form a conjugated diene [1]. Different bioactive compounds, present in wild edible fruits, mainly vitamin C and E, have been reported to have biological activity against lipid peroxidation [2].

The most important biological role of vitamin E is to protect PUFA, other components of cell membranes, and LDL, from oxidation by free radicals [3]. Vitamin E is found primarily within the phospholipid bilayer of cell membranes, and it is particularly effective in preventing lipid peroxidation, limiting the accumulation of high levels of products derived from this process that are associated with numerous diseases and clinical conditions [4]. Its action as an antioxidant is due to the donation of a hydrogen atom to peroxyl radicals of unsaturated lipid molecules, forming a hydroperoxide and a tocopheroxyl radical, which reacts with peroxyl radicals forming other adducts more stable.

Wild food plants, particularly wild fruits, have been an important element in the Mediterranean dietary traditions since the beginning of human occupation of the region.

The consumption of locally grown species is gaining an increasing interest, which also gives an important contribution to local communities' health and welfare [5]. In addition, wild fruits contain higher amount of nutrients and bioactive compounds than many cultivated species, especially those that have been under cultivation for many generations [6-8]. Their traditional consumption, particularly fruits from strawberry-tree (*Arbutus unedo* L.), blackthorn (*Prunus spinosa* L.), hawthorn (*Crataegus monogyna* Jacq.) and blackberry (*Rubus ulmifolius* Schott.), in the Iberian Peninsula as a part of our Mediterranean diet, has been documented in many ethnobotanical surveys [9-10]. All these fruits can be consumed raw (*P. spinosa* fruits must be mature), as well as cooked in jams and included in alcoholic beverages or liqueurs [9-13]. These liqueurs were traditionally used due their digestive properties, especially in the case of *Prunus spinosa* [14-15], but also in the case of blackberries [16-17] or *Crataegus monogyna* [18]. The fruits of the strawberry tree have been popularly used preventively for scurvy [19] or diuretic [13], whereas those of the hawthorn fruits have been regarded with these same properties of its flowers [17-19] and also against colds [20]. Blackthorn fruits have been also mentioned as hypoglycemiant [20] and as an astringent, purgative and diuretic [21]. Finally, the blackberries of *Rubus ulmifolius* have been regarded as antidiarrhoeal [16] and/or against iron deficiency [14].

The present study aims to characterize the bioactive compounds and lipid peroxidation inhibition capacity of wild edible fruits (*Arbutus unedo, Crataegus monogyna, Prunus spinosa* and *Rubus ulmifolius*). Moreover, it seeks the recognition of the nutritional interest of such fruits, which would support traditional consumption and would contribute to the sustainable development in different rural environments of Spain.

2 Material and methods

2.1 Plant material and samples preparation

Fruits of four different wild species were chosen: *Arbutus unedo* L., *Crataegus monogyna* Jacq., *Prunus spinosa* L., and *Rubus ulmifolius* Schott, according to a previous ethnobotanical review [9]. In order to have representative samples, and taking into account the geographical and environmental variability, each species was collected from two different wild populations (of central and western Spain) and during at least two years, from 2007 to 2009. A minimum of 500 g of fruits were collected from different small trees, packed in plastic recipients and carried to the laboratory in a cold system within the same day. All the samples of the selected species presented a healthy external appearance.

Fresh fruits were homogenized in a laboratory blender (immediately after arriving to the laboratory). Aliquots were taken to analyze physicochemical analysis, vitamin C and organic acids. Fatty acids, tocopherols and antioxidant assays were performed in freeze-dried samples previously homogenized from all sample collected to get a representative sample of each species (stored at -20 ºC until analysis). Triplicate subsamples were taken for each analytical procedure.

2.2 Standards and reagents

The solvents *n*-hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-Scan (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), as well as other individual fatty acid isomers, tocopherol standards (α, β, γ and δ), trolox, L (+)-ascorbic, oxalic, malic, citric and succinic acids were purchased from Sigma (St. Louis, MO, USA). Glutamic acid and L-Cystein were supplied by from Merck (Germany). Racemic tocol, 50 mg/ml, was

purchased from Matreya (PA, USA). All other chemicals and solvents were of analytical grade and obtained from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3 Physicochemical analysis

Dry matter (DM) was determined by desiccation to constant weight at 100 ± 2 ºC following AOAC official methods [22]; pH was measured by a potentiometer (Micro pH-2000, Crison Instrument) over an homogenized sample 1/10 (w/v) in distilled water; titratable acidity (TA) was determined by titration with 0.1 N NaOH until pH of 8.1 was reached.

2.4 Fatty acids

Aliquots of freeze-dried sample (~3 g) were extracted with petroleum ether, using a Soxhlet apparatus, according to AOAC procedures [22], to obtain crude fat, which was subjected to a trans-esterification procedure. Fatty acids were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 ºC and 160 rpm; then 3 mL of deionised water was added, to obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by shaking in vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Millipore.

Individual fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by Barros et al. [7]. Equipment used was a DANI model GC 1000 instrument equipped with a split/splitless injector, a FID (at 260 ºC) and a 50% cyanopropyl-methyl - 50%

phenylmethylpolysiloxane column (Macherey-Nagel, Düren, Germany), 30 m × 0.32 mm ID × 0.25 μm d_f. The oven temperature programme was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/min ramp to 180 ºC, 3ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and maintained for 15 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 out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME (fatty acids methyl ester) peaks from samples with the standards. The results were recorded and processed using CSW DataApex 1.7 software and expressed in relative percentage of each fatty acid.

2.5 Vitamin E (tocopherols)

Tocopherols content was determined in edible fruits following a procedure previously described by Barros et al. [6]. To avoid oxidations, BHT (butylhydroxytoluene) solution in hexane (10 mg/mL; 100 μL) and internal standard (IS) solution in hexane (tocol; 2.0 μg/mL; 250 μL) were added to the samples prior to the extraction procedure. The samples (500 mg freeze dried fruit) were homogenised 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 homogenised (1 min), centrifuged (Centurion K24OR refrigerated centrifuge, 5 min, 6185 rpm) 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, re-dissolved in 1 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 was analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamine II (250 x 4.6 mm) normal phase column from YMC Waters (Japan) operating at 30 °C (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 10 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherols content in the samples were expressed in mg/100 g of fresh weight (fw).

2.6 Vitamin C and organic acids

Vitamin C (ascorbic, AA and dehydroascorbic acid, DHA) as well as individual organic acids (oxalic, glutamic, malic, citric and fumaric acids) were determined based on protocols described and optimized by Sánchez-Mata et al. [23], using High-Performance Liquid Chromatography (HPLC) after extraction with 4.5 % m-phosphoric acid. An aliquot of the extracts were also subjected to reduction with 4 % L-cystein to transform DHAA in AA and analysed the total vitamin C content. DHAA was estimated by the difference between total vitamin C and AA contents. The HPLC equipment used was a liquid chromatograph (Micron Analítica, Madrid, Spain) equipped with an isocratic pump (model PU-II), an AS-1555 automatic injector (Jasco, Japan), a Sphereclone ODS (2) 250 x 4.60, 5 μm Phenomenex column, and a UV-visible detector

(Thermo Separation Specta Series UV100). The mobile phase was 1.8 mM H_2SO_4 (pH = 2.6). For AA analysis a flow-rate of 0.9 ml/min and UV detection at 245 nm was used, whilst conditions for organic acids were 215 nm UV detection and 0.4 ml/min flow rate. All data was analysed using Biocrom 2000 3.0 software. Linear calibration curves were obtained for quantification purposes from solutions with known amounts of all the identified compounds (AA, oxalic, glutamic, malic, citric and fumaric acids), in *m*-phosphoric acid to prevent the oxidation of AA.

2.7 Evaluation of lipid peroxidation inhibition

The lipid peroxidation inhibition was evaluated through two different antioxidant activity assays: β-carotene bleaching inhibition assay and TBARS assay.

2.7.1 Extracts preparation. A fine dried powder (1 g of freeze dried fruit) was extracted by stirring with 40 mL of methanol at 25 °C for 1 h and filtered through Whatman No. 4 filter paper. The residue was then extracted with an additional 40 mL portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210), re-dissolved in methanol at a concentration of 5 mg/ml, and stored at 4 °C for further use.

2.7.2 Inhibition of β-carotene bleaching. The evaluation of β-carotene bleaching inhibition assay is based on the non-specific oxidation of linoleic acid, catalyzed by heat (50 ºC). The addition of an extract containing antioxidants promotes a decolouration delay of β-carotene by the inhibition of the oxidation of linoleic acid. A solution of β-carotene was prepared by dissolving the compound (2 mg) in chloroform (10 mL). Two millilitres of this solution were transferred into a round-bottom flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask shaking

vigorously. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). As soon as the emulsion was transferred to each tube, the zero time absorbance was measured at 470 nm. Then tubes were incubated at 50 °C in a shaking water bath [6]. β-Carotene bleaching inhibition was calculated using the following equation:

β-Carotene bleaching Inhibition ratio (%) = absorbance after 2 h of assay/initial absorbance) × 100. (1)

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.

2.7.3 Thiobarbituric acid reactive substances (TBARS) assay. TBARS assay seeks to assess the reaction of thiobarbituric acid with malondialdehyde, formed from the decomposition of lipid hydroperoxides, lipid substrates from the used pig brain cell homogenates.

Brains cells were obtained from pig (*Sus scrofa*), dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate, which was centrifuged at 3000g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the extracts (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C 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 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm [6]. The inhibition ratio (%) was calculated using the following formula, where A and B were the absorbance of

the control and the extract solution, respectively.

TBARS Inhibition ratio (%) = [(A×B)/A] × 100 (2)

The extract concentration providing 50% of lipid peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract concentration.

2.8. Statistical analysis

Each sample was analysed in triplicate. The results were expressed as means and standard deviations (n=12 or 18, analytical triplicate of 4 or 6 different fruit samples) in the case of physicochemical, vitamin C and organic acids analyses, and n=3 (analytical triplicate of 1 complex sample) for the other analyses. Analysis of variance (ANOVA), followed by Duncan`s test, was conducted using Statgraphics Plus 5.1 software to analyze data at the 95% confidence level. Moreover, correlations and Principal Components Analysis (PCA) were performed using Statgraphics Plus 5.1 software with a multivariable analysis.

3 Results and discussion

3.1 Physicochemical analysis

As shown in Tab. 1, the studied wild edible fruits showed relatively high moisture content, from 57.56 to 75.11 g/100 g in *A. unedo* and *R. ulmifolius*, respectively. Regarding the pH and tritrable acidity, *P. spinosa* was the most acidic wild fruit, with a titratable acidity value of 194.82 mL of NaOH N/10 per 100 g (and a pH around 3.43), followed by *A. unedo*, with an acidity of 92.37 mL NaOH N/10 per 100 g (and a pH of 3.37). These results are similar to the pH values reported by other authors in Portuguese samples of blackthorn (3.92) and strawberry-tree (3.74) fruits, [24-25]. *R. ulmifolius*

fruits showed the lowest tritable acidity value (44.11 mL NaOH N/10 per 100 g) and the highest pH value (5.08).

3.2 Lypophilic phytochemicals

3.2.1 Fatty acids

At least twenty-five individual fatty acids were identified in the wild fruits GC profile (Tab. 2). Regarding saturated fatty acids (SFA), *C. monogyna* and *P. spinosa* were the wild fruits with the highest content (58.51 and 55.08%, respectively), mainly due to the contribution of palmitic acid (PA, C16:0), that was found with values around 30.56 and 23.42%, in both fruits respectively. *R. ulmifolius* gave the lowest amount of PA (C16:0; 7.02%). Morever, for *P. spinosa,* it is also important the amount of eicosanoic acid (C20:0, 7.23%). Barros et al. [7] reported fatty acids content in Portuguese origin blackthorn and strawberry-trees; *P. spinosa* fruits analysed by these authors presented a much lower SFA content (15.16%) with a small amount of PA (6.50 %), whereas wild strawberry-trees, Portuguese and Spanish, presented similar content of SFA and PA than those presented in this study.

Unsaturated fatty acids (MUFA and PUFA) are known as one of the factors associated with a reduced risk of developing cardiovascular disease, inflammatory and autoimmune diseases such as asthma, Crohn's disease and arthritis, and certain cancers, including colon, breast and prostate cancers [26]. In the case of monounsaturated fatty acids (MUFA), *A. unedo* and *R. ulmifolius* presented the highest MUFA contents (24.90 and 23.18%, respectively), being the oleic acid (OA, 18:1n9) the most representative with percentages around 24.82% and 22.62% in both fruits. Barros et al. [7] reported a higher MUFA content for blackthorn fruits from Portugal (58.45%) compared with our

sample (9.22%), and also a higher OA percentage for both wild Portuguese origin fruits (57.58 and 21.01%, respectively).

Moreover, the fruits of *A. unedo* and *R. ulmifolius* presented the highest PUFA content (55.48 and 61.97%), with a good balance of unsaturated fatty acids. The beneficial effects attributed to the n-3 PUFA are due to their anti-aggregate effects in cardiovascular diseases. Therefore, the ratio of dietary α-linolenic acid (ALA) to linoleic acid (LA) is very important from a nutritional point of view [27]. To this respect, the major PUFA in the analyzed samples was LA (C18:2n6), with 48.46% in the case of *R. ulmifolius*. Moreover, ALA (C18:3n3) was found in all the studied wild fruits with a relatively important percentage, as observed in strawberry-tree fruits (31.26%; Tab. 2). Gamma linolenic acid (GLA, C18:n6), whose absence in the diet produces a wide variety of diseases, such as diabetic neuropathy, rheumatoid arthritis, cardiovascular, reproductive and autoimmune disorders [28], was also found in all samples, except for *Prunus spinosa*. Comparing with the PUFA percentage reported in wild Portuguese blackthorn and strawberry-tree fruits [7], similar values were obtained (26.40 and 58.28%, respectively). Moreover, both wild fruits presented the same major PUFA, ALA and LA, respectively.

A low n-3/n-6 ratio, as observed in today´s Western diet, promotes the pathogenesis of many diseases, including cardiovascular diseases, cancer, osteoporosis, inflammatory and autoimmune diseases, whereas increased levels of omega-3 (n-3) PUFA (a higher n-3/n-6 ratio), exert suppressive effects [26]. This ratio and PUFA/SFA have been calculated for all samples analyzed (Tab. 2). As it can be observed, all the samples presented a good ratio PUFA/SFA, higher than 0.45, being *R. ulmifolius* (4.16) and *A. unedo* (2.85) the ones with the highest ratio. These results are interesting, as diets rich in

PUFA and low in SFA have been shown to be cardio-protective [29]. Our samples also presented a good n-3/n-6 ratio, with n-3/n-6 > 1 in three of the samples (*A. unedo*, *C. monogyna* and *P. spinosa*).

3.2.2 Vitamin E (tocopherols)

The four tocopherol isoforms (α, β, γ and δ- tocopherols) were identified in all the analysed wild edible fruits, except for *P. spinosa,* in which δ-tocopherol was not detected (Tab. 3). As shown in the table, *R. ulmifolius* presented the highest total tocopherols content (13.48 mg/100 g) with similar contents of α, γ and δ-tocopherols (3.38, 3.17 and 3.69 mg/100g, respectively), followed by *P. spinosa* (5.41 mg/100 g) whose major isoform was α-tocopherol (5.23 mg/100 g; 96.6% of total tocopherols content). *A. unedo* and *C. monogyna* gave percentages of α-tocopherol around 89.94 and 85.45%, respectively.

Pande and Akoh [30] reported tocopherols composition of other *Crataegus* species, describing lower total tocopherols content (1.05 mg/100 g); in agreement with our data, the major isoform was α-tocopherol, but in lower amounts (1.0 mg/100 g), whereas β-tocopherol was not detected. Barros et al. [7] reported tocopherols content in some wild Portuguese species, such as *P. spinosa* and *A. unedo*. Though their data were reflected in dw, only the Portuguese samples of strawberry-tree fruits presented a higher total tocopherols content (23.46 mg/100 g dw, equivalent to 9.45 mg/100g fw) than the Spanish one. Similarly to our data, α-tocopherol was the major isoform in both wild fruits (7.18 and 21.98 mg/100 g dw, respectively), which is the isoform with the highest vitamin E activity [31]. Nevertheless, Barros et al. [7] did not report δ-tocopherol in their samples of strawberry-tree fruits. The analysed wild fruits, due to their high content of α-tocopherol, can contribute to the lipid peroxidation inhibition effect,

mostly in the case of *P. spinosa* that gave a high α-tocopherol content, as well as in *R. ulmifolius* that presented a high γ-tocopherol content. It has been reported that γ-tocopherol and its physiological metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC), inhibit COX-2-catalyzed formation of PGE2, inducing anti-inflammatory properties [32]. Regarding the other isoforms, *Rubus ulmifolius* presented the highest content in β and δ-tocopherols, with values around 0.24 and 3.69 mg/100 g, respectively. Moreover, δ-tocopherol was found in all the samples, except for *P. spinosa* (Tab. 3).

3.3 Hydrophilic phytochemicals

Vitamin C contributes to inhibit lipid peroxidation, due to its action on the tocopheroxyl radical formed by oxidation of α-tocopherol; this radical is stable enough to allow its reduction by vitamin C (ascorbic acid) to regenerate α-tocopherol, thus avoiding the oxidation of PUFA [33]. The oxidized forms of vitamin C, ascorbyl free radical and dehydroascorbate can be recycled back to ascorbate by glutathione (glutamyl cysteine glycine, GSH) or dehydroascorbate reductase enzyme, as well as provide other organic acids with antioxidant properties [1, 33]. Vitamin C content of the studied wild fruits was quite variable. As shown in Tab. 4, *P. spinosa* presented the lowest value of total vitamin C content (7.73 mg/100 g fw), while *A. unedo* presented the highest one (182.41 mg/100 g fw), being ascorbic acid the main isoform (141.99 mg/100 g). In the other fruits, which presented lower total vitamin C values, the predominant isoform was DHAA, representing in *C. monogyna* and *P. spinosa, the* 92 to 98% of total vitamin C content respectively. *R. ulmifolius* presented both isoforms in equal percentages. Erturka et al. [34] reported similar vitamin C values for Turkish blackthorn, around 7.0 to 8.2 mg/100 g, whereas [35] Paulovicsova et al. reported a slightly higher content (10.31

mg/100 g fw). Barros et al. [7] in a Portuguese sample reported values of 15.26 mg/100 g of dw (5.90 mg/100 g fw), slightly lower than our data. However, our vitamin C data for strawberry-tree fruits are considerably higher than those reported by Barros et al. [7] for their Portuguese samples (15.07 mg/100 g dw, equivalent to 6.07 mg/100 g fw). In the case of the analysed *Crataegus monogyna* fruits, Pande and Akon [30] reported similar values for vitamin C content (as ascorbic acid, 13.6 mg/100 g fw) in Turkish fruits of other *Crataegus* species.

In addition to ascorbic acid, other organic acids (oxalic, malic, citric, fumaric and succinic) were detected and quantified in the plants analysed. These compounds are cids metabolically related with ascorbic acid [36] and with antioxidant capacity, since they have been suggested to contribute to tocopherols recovery in redox systems [33; 37]. Malic acid was the major organic acid in all the studied wild fruits, ranging from 260.45 to 323.94 mg/100 g of fw, for *A. unedo* and *C. monogyna*, respectively (Tab. 4). There are only a few studies on the chemical composition of non-volatile organic acids fraction of wild fruits, in most of them malic acid was reported as the major organic acid [30, 38-39]. Related to oxalic acid was found in all the samples in similar values, being the highest content detected in *R. ulmifolius* (98.13 mg/100 g fw), while in *C. monogyna* was found to be the lowest one (57.40 mg/100 g). Regarding citric acid, it was detected in all the studied samples (with the exception of *A. unedo* fruits), with values between 46.63 and 68.77 mg/100 g for *R. ulmifolius* and *C. monogyna*, respectively (Tab. 4). These values were higher than those reported by Pande and Akon [30] for this organic acid in Turkish origin fruits (17.1 mg/100 g fw). Fumaric acid was quantified in all samples evaluated, being *A. unedo* the one with the highest content (0.73 mg/100 g), Alarcão-E-Silva et al. [38] reported similar results, whereas in Turkish origin *Crataegus* was not detected [30]. In the case of succinic acid, it was only

characterized in *C. monogyna* and *R. ulmifolius* with results quite variable (6.17 and 64,15 mg/100 g, respectively).

3.4 Lipid peroxidation inhibition

Lipid peroxidation is generally induced by a hydroxyl radical, creating a chain of oxidative reactions in the lipid bilayer of cell membranes mainly composed of PUFA and, therefore, vulnerable to suffer attacks by free radicals that result in lipid peroxidation [40]. As Tab. 3 and Fig. 1 show, *R. ulmifolius* which is the species with the highest tocopherols and PUFA content, also showed the highest antioxidant activity measured by inhibition of β-carotene bleaching (EC_{50} 21.00 μg/mL) as well as good results for TBARS assay (EC_{50} 25.3 μg/mL). *P. spinosa* is the species with the better lipid peroxidation inhibition capacity measured by TBARS essay (22.6 μg/mL). Otherwise, this species and *A. unedo* presented the lowest lipid peroxidation inhibition in the β-carotene bleaching inhibition assay (86.00 and 98.00 μg/mL, respectively).

To establish the possible influence of the analysed bioactive compounds in the biological capacity (lipid peroxidation inhibition) of the wild edible fruits considered in this study, a correlation analysis between all these parameters was performed. Results showed that total tocopherol content is negatively and strongly correlated with MUFA ($r = 0.968$, $p = 0.006$) and δ-tocopherol with PUFA ($r = 0.977$, $p = 0.004$), MUFA and γ-tocopherol ($r = 0.977$ and $p = 0.004$), PUFA and total tocopherols ($r = 0.945$ and $p = 0.015$), and PUFA and δ-tocopherol ($r = 0.952$ and $p = 0.012$). On the other hand, lipid peroxidation inhibition was positively correlated with SFA content. The correlations observed between lipophilic compounds (tocopherols, MUFA and PUFA) and antioxidant properties of the wild fruits, were significant and negative for both assays, mainly between γ, δ and total tocopherols, justifying the tocopherols influence in the

lipid peroxidation prevention, specially established by β-carotene bleaching inhibition assay.

There is a wide number of studies on antioxidant properties of wild fruits, measured using DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), ORAC (Oxygen Radical Absorbance Capacity) and reducing power (Fe^{3+} to Fe^{2+}) assays, but only a very few regarding lipid peroxidation inhibition [6-7, 25]. Barros et al. [6-7] reported higher EC_{50} values for Portuguese blackthorn and strawberry-tree fruits measured by β-carotene bleaching inhibition (986 and 774.99 μg/mL, respectively) and TBARS (153.86 and 94.27 μg/mL, respectively) assays, which implies a lower ability against lipid peroxidation inhibition. Ganhão et al. [25] reported lipid peroxidation inhibition measured only by TBARS assay, but it is not possible to compare with our data due to the different units used to express the results (μm TEAC/g). They reported that the greatest antioxidant efficacy in this assay was displayed by *A. unedo*, followed by *C. monogyna* and *R. ulmifolius* ($p > 0.05$). Those results were not in agreement with our data, because in our samples *A. unedo* was the species, which presented the lowest lipid peroxidation inhibition capacity.

Pharmacological studies also demonstrated that wild edible fruits, such as *Crataegus* sp. (fruits and flowers), could decrease the level of cholesterol in serum [41] and inhibit platelet aggregation [42]. Evidences show that *Crataegus* extracts have antioxidant effects *in vitro* or *in vivo*, scavenges superoxide anion, hydroxyl radical, and hydrogen peroxides, and inhibits lipid peroxidation [43]. *In vivo* experiments show that supplementats with hawthorn fruits (2%) significantly increased serum α-tocopherol in rats; serum α-tocopherol in the hawthorn fruit-supplemented group was increased by

20% compared with that of the control rats. Therefore, *Crataegus* extracts (from fruits) increased the concentration of α-tocopherol and inhibited the oxidation of human LDL [44]. Our results revealed a high and interesting lipid peroxidation inhibition, and in further studies it would be of great interest to evaluate the ability of these wild species against lipid peroxidation and oxidation of LDL using *in vivo* assays, demonstrating its interest in the prevention of cardiovascular disease, as previously mentioned for *Crataegus* sp.

4 Principal components analysis (PCA)

A multivariate analysis was applied to characterize and classify the wild edible fruits according to chemical and antioxidant characteristics. A principal component analysis (PCA) was performed to reduce the multidimensional structure of the data, providing a three-dimensional map to explain the observed variance. The three components of the PCA performed explain 100% of the total variance. Correlation coefficients between the variables and the three principal components of the analysis were obtained. The first principal component (52.3% of total variance) is highly correlated with dry matter and in a minor degree with ascorbic acid, total vitamin C and β-carotene bleaching inhibition assay (positive correlations). It is also negative correlated with β-tocopherol, MUFA and PUFA. The second principal component (33% of total variance) separates the fruits according to acidity and SFA. Malic acid followed by citric acid was the most important acids that influence the formation of the second principal component. Fumaric acid also contributes to the formation of this second component showing an important and negative correlation. The third component (14.68% of total variance) is highly correlated to oxalic acid and α-tocopherol variables.

All the fruit samples are plotted on the reduced space of the two first principal components as it is shown in Fig. 2. As can be observed, *A. unedo* and *R. ulmifolius* are characterized by the first principal component, positive and negative, respectively (3.02 and -4.11), while *P. spinosa* is characterized by second principal component (3.29), being *C. monogyna* characterized by the third component with a correlation coefficient of -2.55.

This statistical analysis show that lipid peroxidation inhibition is well described by PCA, being mainly characterized by the first principal component. Therefore, we can affirm that this axis is an indicator of healthy compounds and antioxidant fruits status, characterizing fruits with a high positive impact in health, as it is the case of *Arbutus unedo* fruits.

4. Conclusions

Chemical and nutritional values justify the need to preserve their traditional use, as an alternative to the variety of fruits currently available. In this sense, research on wild edible species should be encouraged, both from the nutritional and phytotherapeutical points of view. We can conclude that wild edible fruits can be a good alternative to the currently available range of edible fruits, being potential sources of functional foods, for lipid bioactive compounds for dietary supplements. Moreover, this study provides useful and relevant information that justify the tocopherols influence in the lipid peroxidation prevention with a strong correlation observed between the lipophilic bioactive compounds and the antioxidant activity assays evaluated; this correlation demonstrate that tocopherols can be used as functional ingredients in food technology, being useful in the prevention of impaired lipid foods high in oils or fats.

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Table 1. Physicochemical characteristics of wild edible fruits. Mean ± SD, n=18. In each row, different letters mean statistically significant difference ($p \leq 0.05$).

	Arbutus unedo	*Crataegus monogyna*	*Prunus spinosa*	*Rubus ulmifolius*
Moisture [g/100 g fw]	57.56 ± 9.27 [a]	69.06 ± 6.62 [a]	68.06 ± 2.44 [a]	75.11 ± 3.58 [a]
pH	3.37 ± 0.11 [a]	4.18 ± 0.38 [b]	3.43 ± 0.14 [a]	5.08 ± 0.32 [c]
Titratable acidity [mL NaOH/100g fw]	92.37 ± 32.83 [a]	56.87 ± 26.23 [a]	194.82 ± 73.23 [b]	44.11 ± 32.89 [a]

Table 2. Fatty acids in wild edible fruits. Mean ± SD, n = 3. In each row, different letters mean statistically significant difference ($p \leq 0.05$).

	Arbutus unedo	*Crataegus monogyna*	*Prunus spinosa*	*Rubus ulmifolius*
C6:0	0.11 ± 0.09	0.11 ± 0.01	0.22 ± 0.02	0.34 ± 0.03
C8:0	0.06 ± 0.03	0.16 ± 0.01	0.33 ± 0.01	0.04 ± 0.00
C10:0	0.06 ± 0.04	0.22 ± 0.01	0.49 ± 0.02	0.09 ± 0.00
C11:0	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	nd
C12:0	0.92 ± 0.12	2.42 ± 0.06	2.17 ± 0.06	0.69 ± 0.05
C13:0	nd	nd	0.05 ± 0.00	nd
C14:0	1.63 ± 0.07	1.21 ± 0.00	1.42 ± 0.06	0.25 ± 0.00
C14:1	nd	0.06 ± 0.00	nd	0.02 ± 0.00
C15:0	0.09 ± 0.00	0.22 ± 0.01	0.46 ± 0.01	0.08 ± 0.00
C16:0	10.54 ± 0.15	30.56 ± 0.66	23.42 ± 0.20	7.02 ± 0.13
C16:1	0.08 ± 0.05	0.40 ± 0.02	1.10 ± 0.01	0.15 ± 0.00
C17:0	0.34 ± 0.00	0.77 ± 0.02	1.03 ± 0.00	0.27 ± 0.01
C17:1CIS-10	nd	nd	0.07 ± 0.00	0.06 ± 0.00
C18:0	4.34 ± 0.21	3.88 ± 0.07	14.12 ± 0.09	3.39 ± 0.19
C18:1n9c+t	24.82 ± 0.00	10.66 ± 0.52	8.05 ± 0.02	22.62 ± 0.97
C18:2n6c	24.22 ± 0.15	10.52 ± 0.27	14.18 ± 0.09	48.56 ± 0.91
C18:3n3	31.26 ± 0.04	16.10 ± 0.35	10.58 ± 4.84	13.28 ± 0.22
C18:3n6	0.68 ± 0.02	2.90 ± 0.12	nd	1.45 ± 0.00
C20:0	nd	nd	7.31 ± 0.13	0.36 ± 0.02
C20:1CIS-11	nd	3.06 ± 0.13	0.92 ± 0.42	0.08 ± 0.02
C20:3n3+C21:0	nd	nd	nd	nd
C22:0	0.44 ± 0.05	4.67 ± 0.03	2.20 ± 0.22	0.73 ± 0.00
C22:2 CIS 13,16	nd	0.67 ± 0.01	nd	nd
C23:0	nd	nd	nd	0.05 ± 0.00
C24:0	0.40 ± 0.26	13.32 ± 1.79	1.71 ± 0.05	0.47 ± 0.04
Total fat (%)	1.11	1.18	0.85	1.41
SFA (% of total FA)	19.62 ± 0.04 ^{a}	58.51 ± 3.56 ^{b}	55.08 ± 0.09 ^{b}	14.90 ± 0.17 ^{a}
MUFA (% of total FA)	24.90 ± 0.06 ^{b}	11.13 ± 0.55 ^{a}	9.22 ± 0.02 ^{a}	23.18 ± 0.94 ^{b}
PUFA (% of total FA)	55.48 ± 0.11 ^{b}	30.36 ± 0.50 ^{a}	25.69 ± 4.31 ^{a}	61.97 ± 1.11 ^{c}
PUFA/SFA (% of total FA)	2.85 ± 0.03 ^{c}	0.54 ± 0.03 ^{a}	0.64 ± 0.02 ^{b}	4.16 ± 0.12 ^{d}
n-3/n-6	1.29 ± 0.07 ^{b}	1.82 ± 0.02 ^{d}	1.51 ± 0.02 ^{c}	0.27 ± 0.00 ^{a}

SFA=Saturated fatty acids

MUFA= Monounsaturated fatty acids

PUFA=Poliunsaturated fatty acids

FA=Fatty acids

nd= not detected

Table 3. Tocopherols content and lipid peroxidation inhibition capacity (EC_{50} values) in wild edible fruits. Mean ± SD, n = 3. In each row, different letters mean statistically significant difference ($p \leq 0.05$)

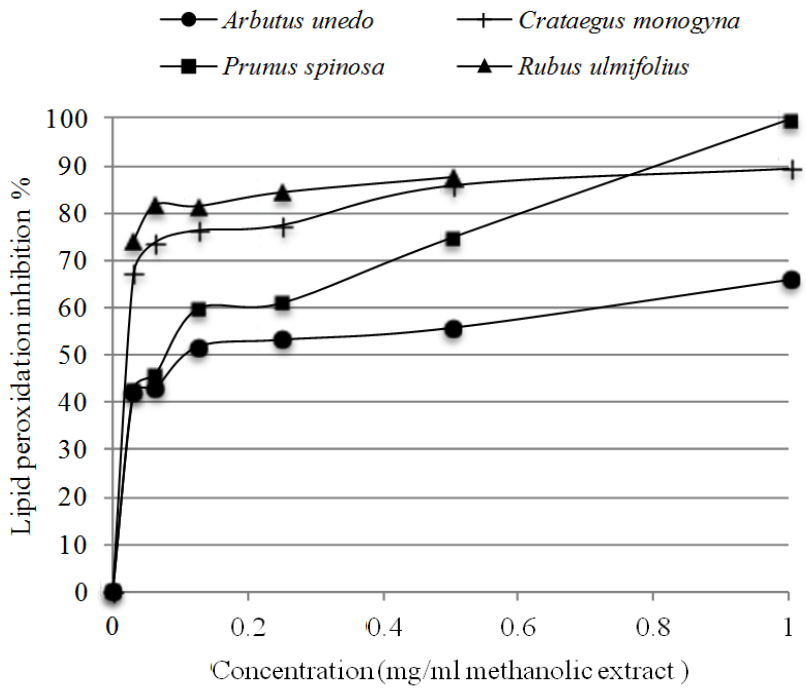
Lypophilic phytochemicals	*Arbutus unedo*	*Crataegus monogyna*	*Prunus spinosa*	*Rubus ulmifolius*
Total tocopherols [mg/100 g fw]	3.88 ± 0.09 [a]	3.37 ± 0.18 [a]	5.41 ± 0.21 [a]	13.48 ± 3.44 [b]
α-tocopherol [mg/100 g fw]	3.49 ± 0.02 [a]	2.88 ± 0.14 [a]	5.23 ± 0.18 [b]	3.38 ± 1.46 [ab]
β-tocopherol [mg/100 g fw]	0.03 ± 0.01 [a]	0.15 ± 0.01 [b]	0.08 ± 0.01 [ab]	0.24 ± 0.10 [c]
γ-tocopherol [mg/100 g fw]	0.26 ± 0.00 [a]	0.17 ± 0.01 [a]	0.09 ± 0.02 [a]	3.73 ± 1.66 [b]
δ-tocopherol [mg/100 g fw]	0.10 ± 0.07 [a]	0.16 ± 0.03 [a]	nd	3.69 ± 1.65 [b]
EC_{50} values [μg/mL]				
β-carotene bleaching inhibition	98.00 ± 1.00 [b]	24.80 ± 0.00	86.00 ± 1.00	25.30 ± 0.00 [a]
TBARS essay	25.00 ± 0.00 [b]	23.00 ± 0.00	22.6 ± 0.00 [a]	21.00 ± 0.00 [b]

nd = not detected.

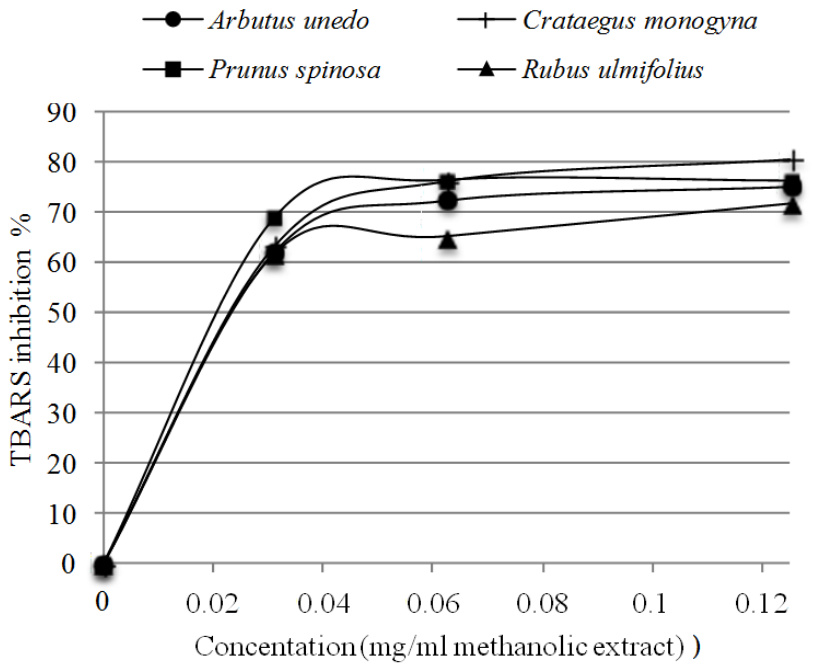
Table 4. Total vitamin C and organic acids content of wild edible fruits [mg/100 g fresh weight]. Mean ± SD, n=18. In each row, different letters mean statistically significant difference ($p \leq 0.05$).

	Arbutus unedo	*Crataegus monogyna*	*Prunus spinosa*	*Rubus ulmifolius*
Total vitamin C	182.41 ± 54.64 [b]	15.19 ± 6.65 [a]	7.73 ± 2.83 [a]	17.09 ± 6.33 [a]
Ascorbic acid	141.99 ± 40.34 [b]	1.51 ± 1.18 [a]	0.91 ± 0.83 [a]	7.41 ± 3.75 [a]
Dehydroascorbic acid	57.43 ± 34.42 [b]	14.89 ± 5.58 [a]	7.15 ± 2.47 [a]	9.71 ± 4.55 [a]
Oxalic acid	96.53 ± 43.05 [a]	57.40 ± 26.94 [a]	95.55 ± 55.29 [a]	98.13 ± 32.06 [a]
Malic acid	260.45 ± 41.63 [a]	323.94 ±135.10 [ab]	291.52 ± 42.12 [ab]	179.49 ± 122.76 [a]
Citric acid	nd	68.77 ± 24.17 [b]	54.35 ± 10.33 [a]	46.63 ± 28.78 [a]
Fumaric acid	0.73 ± 0.26 [b]	0.31 ± 0.09 [ab]	0.19 ± 0.09 [a]	0.56 ± 0.25 [ab]
Succinic acid	nd	6.17 ± 4.13 [a]	nd	64.15 ± 16.87 [b]

nd (not detected).



A



B

Figure 1. Antioxidant properties of wild edible fruits using different assays: A) β-carotene bleaching inhibition and B) TBARS formation inhibition.

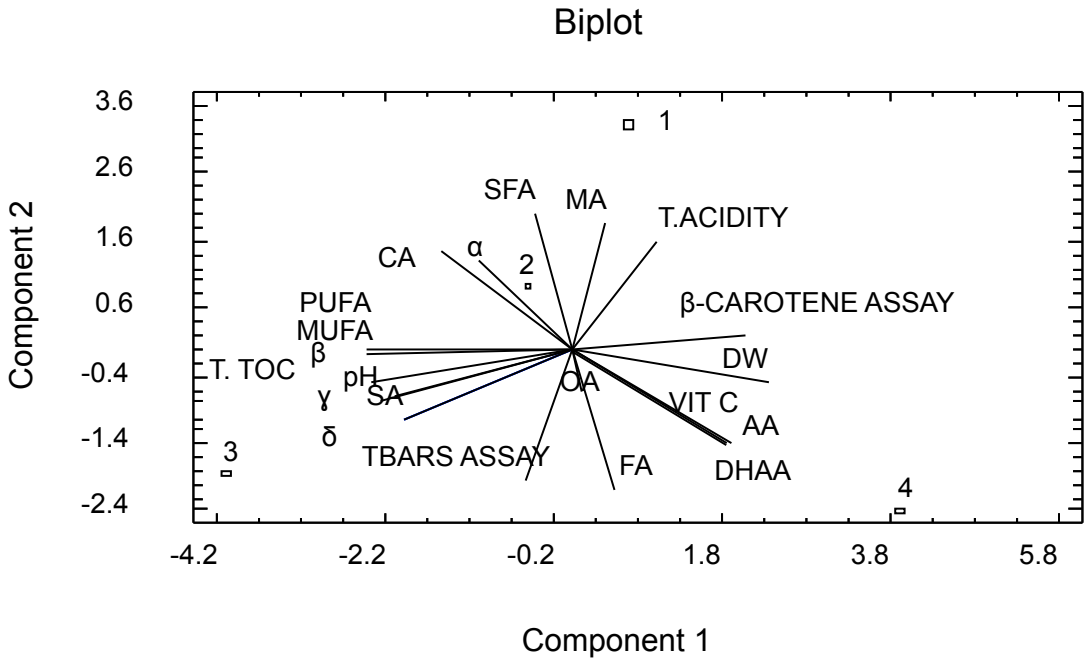


Figure 2. Principal component analysis (PCA) projection of two first principal components. Wild fruits: (1) *Prunus spinosa*, (2) *Crataegus monogyna*, (3) *Rubus ulmifolius*, (4) *Arbutus unedo*. Parameters: DW (dry weight); T. ACIDITY (Tritable acidity); AA (ascorbic acid); DHAA (Dehydroascorbic acid); VIT C (Vitamin C); OA (oxalic acid), CA (Citric acid); FA (fumaric acid); MA (malic acid); SA (succinic acid); SFA (Saturated fatty acids); MUFA (Monounsaturated fatty acids); PUFA (Polyunsaturated fatty acids); α (α-tocopherol); β (β-tocopherol); γ (γ-tocopherol); δ (δ-tocopherol); T. TOC (Total tocopherol); β-CAROTENE ASSAY (β-Carotene Bleaching inhibition Assay); TBARS assay (Lipid peroxidation inhibition using thiobarbituric acid reactive substances assay).