Characterization of Phenolic Compounds in Wild Fruits from Northeastern Portugal

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Abstract

This study aimed to analyse the phenolic composition of wild fruits of *Arbutus unedo* (strawberry-tree), *Prunus spinosa* (blackthorn), *Rosa canina* and *Rosa micrantha* (wild roses). Analyses were performed by HPLC-DAD-ESI/MS. *Prunus spinosa* fruits presented the highest concentration in phenolic acids (29.78 mg/100 g dry weight), being 3-*O*-caffeoylquinic acid the most abundant one, and flavone/ols (57.48 mg/100 g), among which quercetin3-*O*-rutinoside (15.63 mg/100 g) was the majority compound. (+)-Catechin was the most abundant compound in *A. unedo* (13.51 mg/100 g) and *R. canina* (3.59 mg/100 g) fruits. *Arbutus unedo* fruits presented the highest concentration in flavan-3-ols (36.30 mg/100 g). Cyanidin 3-*O*-glucoside was found in all the studied fruits, being the major anthocyanin in most of them, with the exception of *P. spinosa* samples, in which cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside predominated; *Prunus spinosa* fruit presented the more complex anthocyanin profile among the analysed fruits and also the highest anthocyanin concentrations, which was coherent with its greater pigmentation. All in all, *P. spinosa* presented the highest levels of phenolic acids and flavonoids, including anthocyanins, flavonols and flavones, although no flavan-3-ols could be identified in its fruits. The present study represents a contribution to the chemical characterization of phenolic compounds from wild fruits with acknowledged antioxidant activity and traditionally used for several folk medicinal applications.

Keywords: Wild fruits; Flavonols; Flavon-3-ols; Anthocyanins; HPLC-DAD-ESI/MS.

1. Introduction

Plant polyphenols are a wide group of secondary metabolites and are a common component of our diet. They are widely distributed and can range from simple molecules, such as phenolic acids, to complex molecules with numerous phenolic groups, e.g. acylated flavonoid glycosides, proanthocyanidins or oligomeric hydrolysable tannins. Polyphenols occur primarily in conjugated form, linked to sugars moieties, but also to other compounds, such as carboxylic and organic acids, amines, lipids and even to other polyphenols (Bravo, 1998).

Flavonoids represent the most common and widely distributed group of plant phenolics, and can be further divided into classes including flavones, flavonols, flavanones, flavanols, anthocyanins and isoflavonoids (Veitch & Grayer, 2011). Anthocyanins are one group of widespread natural phenolic compounds present in many flowers, fruits, and vegetables and are responsible for their orange, red, and blue color. They are nontoxic, water-soluble compounds and are of great interest in nutrition and medicine because of their potent antioxidant capacity (Garcia-Alonso et al., 2005), ability to regulate adipocytokine gene expression (Tsuda, Ueno, Yoshikawa, Kojo, & Osawa, 2006), and therefore, possible protective effects on human health (He & Giusti, 2010; Tsuda, 2012). They are also used in dyes industry to replace synthetic pigments by natural ones (Pina, Melo, Laia, Parola, & Lima, 2012).

The interest of plant polyphenols derives from the evidence of their potent antioxidant activity and their wide range of pharmacologic properties including anti-inflammatory, antiallergic and antibacterial activities (Bravo, 1998). Furthermore, epidemiological studies have shown a correlation between increased consumption of fruits and vegetables (associated to flavonoid and other antioxidants) and reduced risk of diseases mediated by oxidative stress such as cardiovascular diseases (Hertog, Feskens,

Hollman, Katan, & Kromhout, 1994, Hertog et al., 1995; He, Nowson, & MacGregor, 2006), certain types of cancer (Hertog et al., 1995). The antioxidant properties of phenolic acids and flavonoids have been related to their redox properties and chemical structures, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, some of them display a metal chelation activity, which hinders transition metals from acting as oxidation promoters (Rice-Evans, Miller, & Paganga, 1997).

Exotic or unusual foods, such as the fruits of *Arbutus unedo* (strawberry-tree), *Prunus spinosa* (blackthorn), *Rosa canina* (dog rose) and *Rosa micrantha* (wild rose) may have great potential for food industries as a source of colors and flavors, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods.

The fruits of *A. unedo* are eaten raw or made in liqueurs. Moreover, the bark or roots decoctions are used as anti-inflammatory, laxative, carminative, digestive, odontalgic and cardiotonic (Novais, Santos, Mendes, & Pinto-Gomes, 2004; Salgueiro, 2004; Carvalho, 2010; Camejo-Rodrigues, 2006). *Prunus spinosa* 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; they have also been used as astringent, diuretic and purgative (Novais et al., 2004; Salgueiro, 2004; Camejo-Rodrigues, 2006; Carvalho, 2010). *Rosa canina* fruits are eaten raw as snacks and possess prophylactic and therapeutic activities against a wide range of ailments, including the inflammatory disorders arthritis, rheumatism, gout, colds and gastrointestinal disorders (Rein, Kharazmi, & Winther, 2004; Orhan, Hartevioğlu, Küpeli, & Yesilalada, 2007; Carvalho, 2010). Beneficial health and cosmetic properties of *R. micrantha* fruits have been transmitted and exploited by rural people for centuries; they are used to prepare homemade remedies that prevent or heal several human

disorders and animal diseases (Parada, Carrió, Bonnet, & Vallès, 2009; Carvalho, 2010).

Our research group has been interested in the chemical characterization of the mentioned wild fruits traditionally consumed in Portugal by their medicinal and edible properties. Recent phytochemical studies performed by us revealed that they have antioxidant properties and important antioxidant molecules such as tocopherols and carotenoids (Barros, Carvalho, Morais, & Ferreira, 2010; Guimarães, Barros, Carvalho, & Ferreira, 2010). A few works on the phenolic composition of *A. unedo* fruits from Italy (Pawlowska, Leo, & Braca 2006), Portugal (Fortalezas et al., 2010; Tavares et al., 2010; Mendes, Freitas, Baptista, & Carvalho, 2011) and Spain (Pallauf et al., 2008; Ganhão, Estévez, Kylli, Heinonen, & Morcuende, 2010), *P. spinosa* from Spain (Ganhão et al., 2010) and *R. canina* from Norway (Hvattum, 2002), Poland (Fecka, 2009), Serbia (Tumbas et al., 2012) and Spain (Ganhão et al., 2010) are available. Nevertheless, there are not available reports on phenolic composition of *R. micrantha* and of *P. spinosa* and *R. canina* samples from Portugal. In this study we performed an exhaustive characterization of phenolic compounds present in the wild fruits of the four species.

2. Materials and Methods

2.1. Samples

The fruits of *Arbutus unedo* L. (strawberry-tree) from Ericaceae, and the Rosaceae species *Prunus spinosa* L. (blackthorn), *Rosa canina* L. (dog rose) and *Rosa micrantha* Borrer ex Sm. (similar to eglantine rose) were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, North-eastern Portugal. Strawberry-tree berries were collected fully ripened in November 2008; well matured blackthorn and

dog rose hips were gathered in late September 2008. *R. micrantha* overripe hips that is fleshy and soft dark red fruits were collected in late autumn 2009. The ripeness degree of materials was established visually using the informant's criteria of colour and texture, according growth conditions and time of the year. In order to obtain homogeneity the colours of different materials and samples were confirmed with the Royal Horticultural Society Colour Charts Edition V.

Morphological key characters from the Flora Iberica (Castroviejo, 2001 and 2004) were used for plant identification. The fruits with seeds were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and stored in the deep-freezer at -20ºC for subsequent analysis.

For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean ± standard deviation (SD).

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic, acetic and trifluoroacetic acid (TFA) were purchased from Prolabo (VWR International, France). The phenolic compounds standards were from Extrasynthèse (Genay, France). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Analysis of phenolic acids, flavone/ols and flavan-3-ols

Extraction procedure. Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v*) at room temperature, 150 rpm, for 1h. The extract was filtered through Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 ºC

(rotary evaporator Büchi R-210) to remove methanol. For purification, the aqueous phase was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and the purified extract was further eluted with 5 mL of methanol. The methanolic extract was concentrated under vacuum, and re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analyses for phenolic acids and flavone/ols. The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was

programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: caffeic acid (y=617.91x–691.51; R^2=0.9991); chlorogenic acid (y=600.27x–763.62; R^2=0.9998); *p*-coumaric acid (y=884.6x+184.49; R^2=0.9999); gallic acid (y=556.94x–738.37; R^2=0.9988); isorhamnetin 3-*O*-glucoside (y=262.31x–9.8958; R^2=1.000); isorhamnetin 3-*O*-rutinoside (y=327.42x+313.78; R^2=0.9991); kaempferol 3-*O*-glucoside (y=190.75x–36.158; R^2=1.000); kaempferol 3-*O*-rutinoside (y=175.02x–43.877; R^2=0.9999); quercetin 3-*O*-glucoside (y=316.48x–2.9142; R^2=1.000); quercetin 3-*O*-rutinoside (y=222.79x–243.11; R^2=0.9998), taxifolin (y=478.06x+657.33; R^2=0.9987). The results were expressed in mg per 100 g of dry weight (dw).

HPLC-DAD-ESI/MS analyses for flavan-3-ols and galloyl derivatives.

The extracts were analysed using the HPLC system described above with the following conditions thermostatted at 25 °C: (A) 2.5% acetic acid in water, (B) 2.5% acetic

acid/acetonitrile (90:10, v/v) and (C) HPLC-grade acetonitrile. The elution gradient established was 0% to 100% B for 5 min, from 0 to 5% C for 35 min, from 5 to 50 % C for 5 min, isocratic 50% C for 5 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm as preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. The MS using the same equipment described above programmed using the following settings: declustering potential (DP) -40 V, entrance potential (EP) -7 V, collision energy (CE) -20V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -40 V, EP -10 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1400.

The proanthocyanidins present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (1-100 μg/ml) of different standards compounds: catechin ($y=161.23x+177.26$; $R^2=0.9992$); ellagic acid ($y=36.81x+257.13$; $R^2=0.9979$); gallic acid ($y=298.26x–634.14$; $R^2=0.9949$). The results were expressed in mg per 100 g of dry weight (dw).

2.4. Analysis of anthocyanins

Extraction procedure. Each sample (1 g) was extracted with 30 mL of methanol containing 0.5% TFA, and filtered through a Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3

cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol:water (80:20, *v/v*) containing 0.1% TFA. The methanolic extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analyses. The extracts were analysed using the HPLC system described above in the conditions described by García-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón (2010). Separation was achieved on an AQUA® (Phenomenex) reverse phase C_{18} column (5 μm, 150 mm × 4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min and from 30 to 35% for 5 min, at a flow rate of 0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (50-0.25 μg/mL) of different standards compounds: cyaniding 3-*O*-glucoside (y=63027x–153.83; R^2=0.9995), delphinidin 3-*O*-glucoside (y=557274x+126.24; R^2=0.9999) and peonidin 3-*O*-glucoside (y=537017x–71.469; R^2=0.9997). The results were expressed in μg per 100 g of dry weight (dw).

3. Results

The characterization of the phenolic compounds present in the wild fruits was performed by HPLC-DAD-MS analysis, and data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and quantification of phenolic acids, flavone/ols, flavan-3-ols, and anthocyanins are presented in **Tables 1-3**. As an example, the HPLC profiles of flavonols (A), flavan-3-ols (B) and anthocyanins (C) of *A. unedo* fruits, recorded at 370, 280 and 520 nm, respectively, can be observed in **Figure 1**.

3.1. Phenolic acids and flavone/ols

Prunus spinosa fruits were the only ones that presented phenolic acids, all belonging to the hydroxycinnamic acid derivative sub-group, among them, five compounds (peaks 1-4 and 6) were quinic acid derivatives identified according to their UV spectra (λ_{max} at 314-330 nm) and pseudo molecular ions $[M-H]^-$ (*m/z* at 337, 353 and 367, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic acid). Peak assignments of the different hydroxycinnamoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) and the hierarchical keys

previously developed by Clifford, Johnston, Knight, & Kuhnert (2003) and Clifford, Knight, & Kuhnert (2005). Peak 1, the major phenolic compound found in *P. spinosa* fruits, and peak 2 were identified as 3-*O*-caffeoylquinic acid and 3-*p*-coumaroylquinic acid, respectively, as peak 1 yielded deprotonated quinic acid (*m/z* at 191) as base peak and another majority ion corresponding the hydroxycinnamic acid residue at *m/z* 179 [caffeic acid-H]$^-$, and peak 2 presented *m/z* 163 [*p*-coumaric acid-H]$^-$ as base peak, a fragmentation pattern characteristic of 3-acylchlorogenic acids (Clifford et al., 2003, 2005). Similarly, peak 4, with a major ion at *m/z* 193 [*p*-ferulic acid-H]$^-$, was tentatively identified as 3-*O*-feruloylquinic acid taking into account its pseudomolecular ion and fragmentation pattern. Peaks 3 and 6 were easily distinguished from its base peak at *m/z* 173 ([quinic acid-H-H_2O]$^-$), in the case of peak 3, accompanied by a secondary fragment ion at *m/z* 179 with approximately 94% abundance, which allowed their identification as 4-*O*-caffeoylquinic and 4-*p*-coumaroylquinic acids according to the fragmentation patterns described by Clifford et al. (2003, 2005). Peak 8 presented a UV spectra similar to caffeic acid, with λ_{max} around 328 nm, and a pseudo molecular ion [M-H]$^-$ at *m/z* 335, releasing a fragment at *m/z* 179 [caffeic acid-H]$^-$ (-162 mu, loss of a hexosyl moiety); it was tentatively identified as a caffeoyl hexoside.

In all the studied samples, quercetin derivatives (λ_{max} around 354 nm, and an MS^2 fragment at *m/z* 301) were particularly abundant (**Table 1**). Quercetin 3-*O*-rutinoside (peaks: 4- *A. unedo*, 11- *P. spinosa*, 4- *R. canina* and 5- *R. micrantha* in **Table 1**) and quercetin 3-*O*-glucoside (peaks: 6- *A. unedo*, 13- *P. spinosa*, 7- *R. canina* and 8- *R. micrantha*), were found in all the studied fruits. Both were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Quercetin pentoside and quercetin rhamnoside ([M-H]$^-$ at *m/z* 433 and 447, respectively) were also found in all the samples (peaks: 8 and 9- *A. unedo*;

17 and 19- *P. spinosa*; 9 and 11- *R. canina*; 10 and 12- *R. micrantha*). A quercetin hexoside ([M-H]$^-$ at *m/z* 463) was also found in *P. spinosa* (peak 14), *R. canina* (peak 8) and *R. micrantha* (peak 9) fruits. Other detected quercetin glycosides were peaks 9 and 12 in *P. spinosa*, both assigned to quercetin pentosyl-hexosides ([M-H]$^-$ at *m/z* 595); peaks 10 and 16 in *P. spinosa* ([M-H]$^-$ at *m/z* 609), tentatively identified as quercetin rhamnosyl-hexoside and quercetin hexosyl-rhamnoside; peaks 20 and 21 in *P. spinosa*, which were assigned to a quercetin acetylhexoside (pseudomolecular ion [M-H]$^-$ at *m/z* 505) and quercetin acetylrutinoside (pseudomolecular ion [M-H]$^-$ at *m/z* 651); and peaks 5 and 6 in *R. canina* and *R. micrantha* ([M-H]$^-$ at *m/z* 477), respectively, identified as quercetin glucuronides. Peaks 1 and 2 in *A. unedo* fruits were assigned to two quercetin galloylhexoside derivatives. Their identities were assigned based on their pseudomolecular ions and MS^2 spectra, releasing fragments corresponding to quercetin (*m/z* at 301) and to distinct losses of hexosyl (-162 mu), pentosyl (-132 mu), rhamnosyl (-146 mu), glucuronide (-176 mu), rutinoside (-308 mu), acetyl (-42 mu) and galloyl (-152 mu) moieties. In none of them the identity of the sugar and positions of location of the substituent could be established.

Other detected flavonols corresponded to kaempferol, isorhamnetin and myricetin derivatives. Kaempferol 3-*O*-rutinoside (peak 15 in *P. spinosa*) and isorhamnetin 3-*O*-rutinoside (peaks: 8- *P. spinosa*, 10- *R. canina* and 11- *R. micrantha*) were identified in accordance with their retention, mass spectra and UV-vis characteristics by comparison with commercial standards. Peak 5 in *A. unedo* ([M-H]$^-$ at *m/z* 447) was identified as a kaempferol hexoside, and peaks 12 and 13 in *R. canina* and *R. micrantha*, respectively, were identified as kaempferol rhamnosyl-hexosides ([M-H]$^-$ at *m/z* 593). Two myricetin rhamnosides ([M-H]$^-$ at *m/z* 463) were also found in *A. unedo* (peaks 3 and 7) releasing an MS^2 fragment at *m/z* 317 ([M-H-146]$^-$, myricetin, loss of a rhamnosyl moiety).

Peak 7 in *P. spinosa* fruits presented a λ_{max} around 346 nm, which could be associated to a flavonol, and a pseudo molecular ion $[M-H]^-$ at *m/z* 739, presenting a fragment at *m/z* 317 that could correspond to myricetin, so that it was tentatively identified as a myricetin derivative. Peak 5 in this fruit was assigned to a flavone, apigenin pentoside ($[M-H]^-$ at *m/z* 401), releasing an MS^2 fragment at *m/z* 269 ($[M-H-132]^-$, apigenin, loss of a pentosyl moiety).

Other flavonoids detected in *R. canina* and *R. micrantha* were identified as taxifolin (flavanonol) and eriodictyol (flavanone) derivatives, λ_{max} around 290 nm. Peaks 1 and 6 in *R. canina* and 2 and 7 in *R. micrantha* presented a pseudo molecular ion $[M-H]^-$ at *m/z* 435, releasing a fragment at *m/z* 303 $[taxifolin-H]^-$ (-132 mu, loss of a pentosyl moiety) and were tentatively identified as taxifolin pentosides. Peaks 2 and 3 *R. canina* and 3 and 4 in *R. micrantha* presented a pseudo molecular ion $[M-H]^-$ at *m/z* 449, releasing a fragment at *m/z* 287 $[eriodictyol-H]^-$ (-162 mu, loss of a hexosyl moiety) and were tentatively identified as eriodictyol hexosides.

Prunus spinosa fruits presented the highest concentration in phenolic acids (29.78 mg/100 g), especially due to 3-*O*-caffeoylquinic acid, the most abundant phenolic acid, and flavone/ols (57.48 mg/100g), in which quercetin 3-*O*-rutinoside (15.63 mg/100g) was the main compound. *Arbutus unedo* fruits presented quercetin 3-*O*-glucoside as the majority flavonol (2.34 mg/100 g).

Taxifolin pentoside was the majority flavanonol derivative in *R. canina* and *R. micrantha* (1.18 and 2.68 mg/100 g, respectively). *Rosa micrantha* (11.16 mg/100 g) presented higher amount of total flavonoids (flavonols, flavanone and flavanonol) compared to *R. canina* (5.50 mg/100 g).

3.2. Flavan-3-ols and galloyl derivatives

Flavan-3-ols (i.e., catechins and proanthocyanidins) were other relevant flavonoids found in *A. unedo*, *R. canina* and *R. micrantha* fruits. Peak 8 in *A. unedo,* peak 11 in *R. canina* and peak 10 in *R. micrantha* were identified as (+)-catechin by comparison of its UV spectra and retention time with a commercial standard. Peak 6 in *R. canina* and Peak 7 in *R. micrantha* presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451, releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to a catechin monomer. This compound was tentatively identified as (+)-catechin-*O*-hexoside, identity that was coherent with its earlier elution (higher polarity) compared with its parent aglycone. Peak 5 in *A. unedo*, peak 7 in *R. canina* and peak 8 in *R. micranta* were identified as procyanidin dimer B1 by comparison of its UV spectra and retention time with a standard available in the laboratory. Similarly, peak 8 in *R. canina* was identified as procyanidin dimer B3. Other signals at *m/z* 577, 865 and 1153, in *A. unedo* (peaks 6, 7, 10 and 11), *R. canina* (peaks 9 and 12) and *R. micrantha* (peaks 12, 17 and 18) can be respectively associated to B-type procyanidin dimers, trimers and tetramers (*i.e.*, (epi)catechin units with C4-C8 or C4-C6 interflavonoid linkages). Peak 3 in *A. unedo* (pseudomolecular ion $[M-H]^-$ at *m/z* 593) was coherent with a proanthocyanidin dimer consisting of one (epi)gallocatechin unit and one (epi)catechin unit. Peaks 13 and 15 in *R. micrantha* ($[M-H]^-$ at *m/z* 851) were coherent with proanthocyanidin trimers formed by two (epi)catechin units and one (epi)afzelechin unit. Pseudomolecular ions $[M-H]^-$ at *m/z* 739, 1027, 1189 and 1351 in *R. canina* (peaks 1, 2, 5 and 10) and in *R. micrantha* (peaks 1, 2, 5, 6, 9 and 16) could correspond to procyanidin dimers or trimers having one to three sugar units attached to them. Also, peaks corresponding to double-charged deprotonated ions $[M-2H]^{2-}$ at *m/z* 819 were detected in *R. canina* (peaks 3 and 4) and *R. micrantha* (peaks 4, 5 and 11), which could

be interpreted as procyanidin tetramers bearing three hexosyl residues in accordance with previous observations by Salminen et al. (2005).

Other compounds detected in the chromatograms obtained for flavan-3-ol analysis were identified as different galloyl derivatives according to their pseudomolecular ions and MS^2 fragmentation behaviour (Table 2). Peaks 1, 2, 4, in *A. unedo* presented pseudomolecular ions $[M-H]^-$ at *m/z* 343, 331 and 325, respectively, releasing a fragment at *m/z* 169 [gallic acid–H]$^-$ (-174, -162 and -156 mu, losses of quinic acid, hexosyl and shikimic acid moieties). These compounds were tentatively identified as galloylquinic acid, galloylhexoside and galloylshikimic acid. Other signals at *m/z* 495 and 477 in *A. unedo* (peaks 9, 12 and 14) can be respectively associated to digalloylquinic and digalloylquinic shikimic acids, respectively. These galloyl derivatives were already described in *A. unedo* by by Pawlowska et al. (2006), Mendes et al. (2011) and Tavares et al. (2010).

Peak 13 in *A. unedo* showed a spectra UV similar to ellagic acid, and was identified as an ellagitannin. This compound was assigned as strictinin ellagitannin according to its pseudomolecular ion ([M-H]- at *m/z* 633) and fragmentation pattern as described by Fortalezas et al. (2010), Tavares et al. (2010) and Mendes et al. (2011).

Peak 1 in *R. micrantha* (**Table 1**) presented λ_{max} around 278 nm and was identified as methyl gallate hexoside ($[M-H]^-$ at *m/z* 345). A similar compound was already reported in rose hip extracts by Hvattum (2002) and further identified by Fecka (2009) as methylgallate 3-*O*-β-glucoside.

(+)-Catechin was the most abundant flavan-3-ol found in *A. unedo* (13.51 mg/100 g) and *R. canina* (3.59 mg/100 g), and also was a prominent compound in *R. micrantha* (2.90 mg/100 g), although a glycosylated proanthocyanidin dimer (peak 6, 4.93

mg/100g) was the most abundant flavan-3-ol in this fruit. *A. unedo* fruits presented the highest concentration in proanthocyanidins (36.30 mg/100 g dw).

3.3. Anthocyanins

The anthocyanin profile obtained for *P. spinosa* fruits, consisting of eight compounds, was more complex that those found in the other fruits. Only one anthocyanin was detected in *R. canina* and *R. micrantha* fruits, whereas three anthocyanins were detected in *A. unedo* fruits. The analytical characteristics, identities and concentrations of the anthocyanins found in the different samples are presented in **Table 3**. Cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside were positively identified by comparison with standards. The identity of cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were also confirmed by comparison of their chromatographic and UV and mass spectral characteristics with data in our library. Peaks 5 and 6 of *P. spinosa* and peak 3 of *A. unedo* were assigned as cyaniding 3-*O*-pentoside and peonidin 3-*O*-pentoside, respectively, based on their mass spectra, which showed an MS^2 signal at *m/z* 287 (cyanidin; $[M-132]^+$, loss of a pentosyl moiety) and *m/z* 301 (peonidin; $[M-132]^+$, loss of a pentosyl moiety). Peaks 7 and 8 presented pseudomolecular ions $[M-H]^+$ at *m/z* 491 and 505 releasing MS^2 fragments at *m/z* 287 and 301 (cyanidin and peonidin, respectively; $[M-H-42-162]^+$, loss of an acetylhexoside moiety), and were tentatively identified as cyaniding 3-*O*-acetylglucoside and peonidin 3-*O*-acetylglucoside, respectively. Cyanidin 3-*O*-glucoside was found in all the samples, being the major anthocyanin in most of them, with exception of *P. spinosa* fruits in which cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside were the major anthocyanins. These fruits also presented the highest anthocyanin concentrations, which was coherent with their higher pigmentation.

4. Discussion

The chemopreventive effect of polyphenols has often been associated with their antioxidant activity. For example, chlorogenic acids are responsible for beneficial roles of some medicinal plants in the prevention of certain oxidative diseases (Morishita & Ohnishi, 2001). Quercetin is also a very efficient antioxidant (Pietta, 2000) and appears to be active in many diseases such as cancer (Choi et al., 2001), cardiovascular (Yoshizumi et al., 2001), and neurodegenerative (Schroeter, Spencer, Rice-Evans, Williams, 2001) disorders. Derivatives of both these compounds were abundant in the studied fruits.

A range of health related properties are also reported for flavon-3-ols namely antiviral (Shahat et al., 2002), insulin-like (Anderson et al., 2004), antitumor (Miura et al., 2008), anti-inflammatory (Terra et al., 2007), and antioxidant (Maldonado, Rivero-Cruz, Mata, & Pedraza-Chaverri, 2005) activities. B-type trimer showed higher antioxidant activity compared to higher oligomers (Shahat et al., 2002). Compared to resveratrol and ascorbic acid, an A-type procyanidin dimer and trimer had similar or better radical scavenging abilities (Maldonado et al., 2005). Thus, both A- and B-type procyanidins have been reported to exert antioxidant activity.

Anthocyanins have demonstrated that, in addition to their colorful characteristics, they possess some positive therapeutic effects, mainly linked with their strong antioxidant properties (Wang, Cao, & Prior, 1997). Cyanidin-3-*O*-glucoside (kuromanin) has been found to have the highest oxygen radical absorbance capacity (Wang et al., 1997). This compound is the most ubiquitous and it represents the main anthocyanin in the edible parts of several plants (Dugo, Mondello, Errante, Zappia, & Dugo, 2001), being also found in the four fruits studied herein.

The highest phenolic acids (29.78 mg/100 g), flavone/ols (57.48 mg/100 g) and anthocyanins (100.40 μg/100 g) contents were found in *Prunus spinosa* fruits, despite no flavan-3-ols compounds were detected in this sample.

As far as we know, there is no information on the phenolic composition of *R. micrantha* fruits; our group has already characterized the phenolic compounds of flowers from this species (Barros et al., 2013). The total amounts of each phenolic group has been reported for *P. spinosa* fruits from Spain (Ganhão et al., 2010), but the individual profile of this species has not been previously described. Ganhão et al. (2010) also analysed total phenolic compounds in *A. unedo* and *R. canina*. Otherwise, for *A. unedo* and *R. canina*, there are a few publications on their individual profile of phenolic composition but from other countries (Hvattum, 2002; Pawlowska et al., 2006; Pallauf et al., 2008; Fecka, 2009; Fortalezas et al., 2010; Tavares et al., 2010; Mendes et al., 2011; Tumbas et al., 2012).

R. canina fruits from Portugal presented some similarities in the phenolic composition to samples from Norway, Poland and Serbia (Hvattum, 2002; Fecka, 2009; Tumbas et al., 2012). Tumbas et al. (2012) identified in the Serbian sample different phenolic acids (gallic, protocatechuic, caffeic, syringic, coumaric, vanillic, ferulic and ellagic acids) and flavonoid aglycones, such as quercetin, kaempferol and myricetin. In the Portuguese sample these types of flavonols were found but all of them were glycosylated; this could be due to the fact that the extract studied by Tumbas et al. (2012) was an infusion of rosehips, and hydrolyses of the sugar moieties could have occurred. The sample from Serbia presented a lower concentration in phenolic compounds and quercetin (296.5 μg/kg of rosehip tea) was the major compound. As for the samples studied herein, (+)-catechin (3.59 mg/100g dw) was the major phenolic compound found. Samples of *R. canina* from Poland (Fecka, 2009) presented a higher

concentration of phenolic compounds than the one studied here, being methylgallate-3-*O*-β-glucoside and catechin the main phenolic compounds. Furthermore, Fecka (2009) identified the presence of ellagitannins such as tellimagrandin I and II and rugosin A, B, D and E, which were not detected in our study. No quantitative information was provided in the study described by Hvattum (2002) regarding phenolic compounds of *R. canina* sample from Norway. Nevertheless, its phenolic profile was similar to the Portuguese sample here analysed, despite some differences in the identification of flavan-3-ols.

For *A. unedo* fruits there are three different studies on the individual phenolic profile of samples from Italy, Spain and Portugal (Pawlowska et al., 2006; Pallauf et al., 2008; Fortalezas et al., 2010; Tavares et al., 2010; Mendes et al., 2011) and they were all very similar to the one obtained in this work, with exception of Italian *A. unedo* fruits (Pawlowska et al., 2006), for which only gallic acid derivatives and anthocyanins were reported, but not flavonols. The comparison of phenolic compounds quantification was not possible, due to the fact that these authors only quantified the anthocyanins, expressing the results in fresh weight. However, the amounts obtained for Spanish *A. unedo* fruits (Pallauf et al., 2008) were very similar to ours. Mendes et al., 2011, Tavares et al., 2010 and Fortalezas et al., 2010 studied a different sample from Portugal, but did not present any type of quantification.

Previous *in vitro* chemical and biochemical assays demonstrated that the studied fruits have a high antioxidant activity and that this could be correlated to their phenolic composition (Barros et al., 2010; Guimarães et al., 2010; Barros, Carvalho, & Ferreira 2011).

Overall, *Prunus spinosa* fruits presented the highest concentration in phenolic acids and flavone/ols, being 3-*O*-caffeoylquinic acid and quercetin 3-*O*-rutinoside the major compounds. (+)-Catechin was the most abundant compound in *Arbutus unedo* and *Rosa canina* fruits. *A. unedo* fruits presented the highest concentration in flavan-3-ols. Cyanidin 3-*O*-glucoside was found in all the studied fruits, being the major anthocyanin in most of them, with the exception of *P. spinosa,* were cyaniding 3-*O*-rutinoside and peonidin 3-*O*-rutinoside predominated. All in all, *P. spinosa* presented the highest levels of phenolic acids and flavonoids, including anthocyanins, flavonols and flavones, although no flavan-3-ols could be identified in its fruits. The present study represents a contribution to the chemical characterization of phenolic extracts from wild fruits with reported antioxidant activity and traditionally used for several medicinal applications. The studied fruits may have great potential for food industries as a source of colors and flavors, as well as bioactive molecules such as phenolic compounds for dietary supplements or functional foods.

Acknowledgements

The authors are grateful to strategic projects PEst-OE/AGR/UI0690/2011 and PEst-C/QUI/UI0686/2011 for financial support to CIMO and CQ-UM, respectively. R. Guimarães and L. Barros thank to FCT, POPH-QREN and FSE for their grants (SFRH/BD/78307/2011 and SFRH/BPD/4609/2008, respectively). The GIP-USAL is financially supported by the Consolider-Ingenio 2010 Programme (FUN-C-FOOD, CSD2007-00063). M. Dueñas thanks the Spanish "Ramón y Cajal" Programme for a contract.

References

Anderson, R.A., Broadhurst, C.L., Polansky, M.M., Schmidt, W.F., Khan, A., Flanagan, V.P., Schoene, N.W., & Graves, D.J. (2004). Isolation and characterization of polyphenol type-A polymersfrom cinnamon with insulin-like biological activity. *Journal of Agricultural and Food Chemistry*, *52*, 65-70.

Barros, L., Carvalho, A.M., Morais, J.S., & Ferreira, I.C.F.R. (2010). Strawberry-tree, blackthorn and rose fruits: Detailed characterization in nutrients and phytochemicals with antioxidant properties. *Food Chemistry*, *120*, 247-254.

Barros, L., Carvalho, A.M., & Ferreira, I.C.F.R. (2011). Exotic fruits as a source of important phytochemicals: Improving the traditional use of *Rosa canina* fruits in Portugal. *Food Reseach International*, *44*, 2233-2236.

Barros, L., Alves, C.T., Dueñas, M., Silva, S., Oliveira, R., Carvalho, A.M., Henriques, M., Santos-Buelga, C., & Ferreira, I.C.F.R. (2013). Towards antifungal compounds exploring phenolic extracts of wild medicinal flowers from Portugal. Ind. Crops and Prod. 2013, 44, 104-110.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, *56*, 317-333.

Camejo-Rodrigues, J.S. (2006). *Recolha dos 'Saber-Fazer' Tradicionais das Plantas Aromáticas e Medicinais, Concelhos de Aljezur, Lagos e Vila do Bispo*. Associação Aflosul, Bordeira.

Carvalho, A.M. (2010). *Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal*. Biblioteca de Ciencias 35. Madrid: Consejo Superior de Investigaciones Científicas.

Castroviejo S. (coord). (2001). *Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares.* Vol. VI, Rosaceae. Real Jardín Botánico, Madrid: CSIC.

Castroviejo S. (coord). (2004). *Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares.* Vol. IV. Real Jardín Botánico, Madrid: CSIC.

Choi, J.A., Kim, J.Y., Lee, J.Y., Kang, C.M., Kwon, H.J., Yoo, Y.D., Kim, T.W., Lee, Y.S., & Lee, S.J. (2001). Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *International Journal of Oncology*, *19*, 837–844.

Clifford, M.N., Johnston, K.L., Knight, S., & Kuhnert, N.A. (2003). A hierarchical scheme for LC-MSn identification of chlorogenic acids. *Journal of Agriculture and Food Chemical*, *51*, 2900-2911.

Clifford, M.N., Knight, S., & Kuhnert, N.A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. *Journal of Agriculture and Food Chemical*, *53*, 3821-3832.

Dugo, P., Mondello, L., Errante, G., Zappia, G., & Dugo, G. (2001). Identification of anthocyanins in berries by narrow-bore high performance liquid chromatography with electrospray ionization detection. *Journal of Agricultural and Food Chemistry*, *49*, 3987–3992.

Fecka, I. (2009). Qualitative and quantitative determination of hydrolysable tannins and other polyphenols in herbal products from meadowsweet and dog rose. *Phytochemical Analysis*, *20*, 177-190.

Fortalezas, S., Tavares, L., Pimpao, R., Tyagi, M., Pontes, V., Alves, P.M., McDougall, G., Stewart, D., Ferreira, R.B., & Santos, C.N. (2010). Antioxidant properties and neuroprotective capacity of strawberry tree fruit (*Arbutus unedo*). *Nutrients 2*, 214-229.

Ganhão, R., Estévez, M., Kylli, P., Heinonen, M., & Morcuende, D. (2010). Characterization of selected wild mediterranean fruits and comparative efficacy

as inhibitors of oxidative reactions in emulsified raw pork burger patties. *Journal of Agriculture and Food Chemical*, *58*, 8854-8861.

Garcia-Alonso, M., Rimbach, G., Sasai, M., Nakahara, M., Matsugo, S., Uchida, Y., Rivas-Gonzalo, J.C., & Pascual-Teresa, S. (2005). Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. *Molecular Nutrition and Food Research*, *49*, 1112-1119.

García-Marino, M., Hernández-Hierro, J.M., Rivas-Gonzalo, J.C., & Escribano-Bailón, M.T. (2010). Colour and pigment composition of red wines obtained from co-maceration of Tempranillo and Graciano varieties. *Analytical Chimica Acta, 660*, 134-142.

Guimarães, R., Barros, L., Carvalho, A., & Ferreira, I.C.F.R. (2010). Studies on chemical constituents and bioactivity of *Rosa micrantha*: An alternative antioxidants source for food, pharmaceutical, or cosmetic applications. *Journal of Agriculture and Food Chemical*, *58*, 6277-6284.

He, F.J., Nowson, C.A., & MacGregor, G.A. (2006). Fruit and vegetable consumption and stroke: metaanalysis of cohort studies. *Lancet*, *367*, 320-326.

He, J., & Giusti, M. (2010) Anthocyanins: natural colorants with health-promoting propertie. *Annual Review of Food Science and Technology*, *1*, 163-187

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B., & Kromhout, D. (1994). Dietary flavonoids and cancer risk in the Zutphen elderly study. *Nutrition and Cancer*, *22*, 175-184.

Hertog, M.G.L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., & Nedeljkovic, S. (1995). Flavonoid

intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine*, *155*, 381-386.

Hvattum, E. (2002). Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled to electrospray ionization tandem mass spectrometry and diode-array detection. *Rapid Communications in Mass Spectrometry*, *16*, 655-662.

IUPAC. (1976). Nomenclature of cyclitols. *Biochemical Journal*, *153*, 23-31.

Maldonado, P.D., Rivero-Cruz, I., Mata, R., & Pedraza-Chaverri, J. (2005). Antioxidant activity of A-type proanthocyanidins from *Geranium niveum* (Geraniaceae). *Journal of Agricultural and Food Chemistry*, *53*, 1996-2001.

Mendes, L., Freitas, V., Baptista, P., & Carvalho M. (2011). Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. *Food and Chemical Toxicology*, *49*, 2285-2291.

Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., Kanda, T., Ohtake, Y., & Sato, T. (2008). Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. *Carcinogenesis*, *29*, 585-593.

Morishita, H., & Ohnishi M. (2001). Absorption, metabolism and biological activities of chlorogenic acids and related compounds. *Bioactive Natural Products*, *25*, Part F, 919-953.

Novais, H.M., Santos, I., Mendes, S., & Pinto-Gomes, C. (2004). Studies on pharmaceutical ethnobotany in Arrábida Natural Park. *Journal of Ethnopharmacology*, *93*, 183–195.

Orhan, D.D., Hartevioğlu, A., Küpeli, E., & Yesilalada, E. (2007). *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. *Jornal of Ethnopharmacology*, *112*, 394-400.

Pallauf, K., Rivas-Gonzalo, J.C., Castillo, M.D., Cano, M.P., & Pascual-Teresa, S. (2008). Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. *Journal of Food Composition and Analysis*, *21*, 273-281.

Parada, M., Carrió, E., Bonnet M. A., & Vallès, J. (2009). Ethnobotany of the Alt Empordà region (Catalonia, Iberian Peninsula). Plants used in human traditional medicine. *Jornal of Ethnopharmacology*, *124*, 609-618.

Pawlowska, A.M., Leo, M., & Braca, A. (2006). Phenolics of *Arbutus unedo* L. (Ericaceae) fruits: Identification of anthocyanins and gallic acid derivatives. *Journal of Agricultural and Food Chemistry*, *54*, 10234-10238.

Pietta, P.G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*, 1035–1042.

Pina, F., Melo, M. J., Laia, C. A. T., Parola, A. J., & Lima, J. C. (2012) Chemistry and applications of flavylium compounds: a handful of colours. *Chemical Society Reviews*, *41*, 869-908

Rein, E., Kharazmi, A., & Winther, K. (2004). A herbal remedy, Hyben Vital (stand. powder of a subspecies of *Rosa canina* fruits), reduces pain and improves general wellbeing in patients with osteoarthritis—a double-blind, placebo-controlled, randomised trial. *Phytomedicine*, *11*, 383-391.

Rice-Evans, C.A., Miller, N.J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. Review. *Trends in Plant Science*, *2*, 152-159.

Salgueiro, J. (2004). *Ervas, usos e saberes*. Lisboa: MARCA, Associação de Desenvolvimento Local.

Salminen, J.P., Karonen, M., Lempa, K., Liimatainen, J., Sinkkonen, J., Lukkarinen, M., & Pihlaja, K. (2005). Characterisation of proantocyanidin aglycones and glycosides from rose hips by high-performance liquid chromatography-mass spectrometry, and their rapid quantification together with Vitamin C. *Journal of Chromatography A*, *1077*, 170-180.

Shahat, A.A., Cos, P., De Bruyne, T., Apers, S., Hammouda, F.M., Ismail, S.I., Azzam, S., Claeys, M., Goovaerts, E., Pieters, L., Vanden Berghe, D., & Vlietinck, A.J. (2002). Antiviral and antioxidant activity of flavonoids and proanthocyanidins from *Crataegus sinaica*. *Planta Medica*, *68*, 539-541.

Schroeter, H., Spencer, J.P.E., Rice-Evans, C., & Williams, R.J. (2001). Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. *Biochemical Journal*, *358*, 547–557.

Tavares, L., Fortalezas, S., Carrilho, C., McDougall, G.J., Stewart, D., Ferreira, R.B., & Santos, C.N. (2010). Antioxidant and antiproliferative properties of strawberry tree tissues. *Journal of Berry Research*, *1*, 3-12.

Terra, X., Valls, J., Vitrac, X., Merrillon, J.M., Arola, L., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., & Blay, M. (2007). Grape-seed procyanidins act as anti-inflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signalling pathway. *Journal of Agricultural and Food Chemistry*, *55*, 4357-4365.

Tsuda, T. (2012). Dietary anthocyanin-rich plants: Biochemical basis and recent progress in health benefits studies. *Molecular Nutrition and Food Research*, *56*, 159-170

Tsuda, T., Ueno, Y., Yoshikawa, T., Kojo, H., & Osawa, T. (2006). Microarray profiling of gene expression in human adipocytes in response to anthocyanins. *Biochemical Pharmacology*, *71*, 1184-1197.

Tumbas, V.T., Canadanovic-Brunet, J.M., Cetojevic-Simin, D.D., Cetkovic, G.S., Dilas, S.M., & Gillec, L. (2012). Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. *Journal of the Science of Food and Agriculture*, *92*, 1273-1281.

Veitch, N., & Grayer, R. (2011). Flavonoids and their glycosides, including anthocyanins. *Natural Product Reports*, *28*, 1626-1695.

Wang, H., Cao, G., & Prior, R.L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry*, *45*, 304–309

Yoshizumi, M., Tsuchiya, K., Kirima, K., Kyaw, M., Suzaki, Y., & Tamaki, T. (2001). Quercetin inhibits Shc- and Phosphatidylinositol 3-kinase-mediated c-Jun N-terminal kinase activation by angiotensin II in cultured rat aortic smooth muscle cells. *Molecular Pharmacology*, *60*, 656–665.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the phenolic acids and flavone/ols of wild fruits.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
				Arbutus unedo			
1	18.7	354	615	615(100),463(18),301(18)	Quercetin galloylhexoside derivative	DAD/MS	0.57 ± 0.03
2	18.9	352	615	615(100),463(36),301(29)	Quercetin galloylhexoside derivative	DAD/MS	1.31 ± 0.00
3	19.2	-	463	317(100)	Myricetin rhamnoside	DAD/MS	0.23 ± 0.01
4	19.5	348	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	1.70 ± 0.02
5	19.9	354	447	285(100)	Kaempferol hexoside	DAD/MS	0.84 ± 0.14
6	20.4	348	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	2.34 ± 0.01
7	20.8	352	463	317(100)	Myricetin rhamnoside	DAD/MS	0.45 ± 0.01
8	23.7	352	433	301(100)	Quercetin pentoside	DAD/MS	1.32 ± 0.04
9	24.7	350	447	301(100)	Quercetin rhamnoside	DAD/MS	2.10 ± 0.04
					Total		10.86 ± 0.24
				Prunus spinosa			
1	5.2	326	353	191(100),179(98),161(14),135(75)	3-*O*-caffeoylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	22.09 ± 0.11
2	7.1	312	337	191(36),163(100),155(8),119(75)	3-*p*-coumaroylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	0.80 ± 0.01
3	7.5	324	353	191(74),179(94),173(100),161(8),135(75)	4-*O*-caffeoylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	3.41 ± 0.03
4	8.2	326	367	193(100),191(16),173(14),149(25)	3-*O*-feruloylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	1.76 ± 0.04
5	8.5	340	401	269(100)	Apigenin pentoside	DAD/MS	1.32 ± 0.03
6	11.1	312	337	191(3),173(100),163(43),155(12),119(21)	4-*p*-coumaroylquinic acid	Clifford et al. 2003 and 2005/ DAD/MS	0.64 ± 0.00
7	14.3	346	739	625(100),607(7),501(9),475(7),317(14),299(24)	Myricetin derivative	DAD/MS	1.06 ± 0.05
8	15.1	328	335	179(20),161(100),135(49)	Caffeoyl hexoside	DAD/MS	1.07 ± 0.00
9	18.9	350	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	1.36 ± 0.04
10	19.5	356	609	301(100)	Quercetin rhamnosyl-hexoside	DAD/MS	2.22 ± 0.03
11	19.8	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	15.63 ± 0.33
12	20.3	352	595	301(100)	Quercetin pentosyl-hexoside	DAD/MS	6.83 ± 0.29
13	20.7	356	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	1.36 ± 0.02
14	21.2	354	463	301(100)	Quercetin hexoside	DAD/MS	4.70 ± 0.50

15	22.9	350	593	285(100)	Kaempferol 3-*O*-rutinoside	Standard/DAD/MS	1.90 ± 0.05
16	23.8	354	609	447(4),301(100)	Quercetin hexosyl-rhamnoside	DAD/MS	7.17 ±0.42
17	24.0	354	433	301(100)	Quercetin pentoside	DAD/MS	8.84 ± 0.26
18	24.4	358	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.87 ± 0.03
19	25.1	348	447	301(100)	Quercetin rhamnoside	DAD/MS	1.30 ± 0.02
20	25.8	356	505	463(5),301(100)	Quercetin acetylhexoside	DAD/MS	1.90 ± 0.05
21	29.8	348	651	609(15),301(100)	Quercetin acetylrutinoside	DAD/MS	1.00 ± 0.01
					Total phenolic acids		29.78±0.17
					Total flavone/ols		57.48±0.74
					Total		87.25 ± 0.91
				Rosa canina			
1	15.9	292	435	303(24),285(100)	Taxifolin pentoside	DAD/MS	1.18 ± 0.03
2	16.9	294	449	287(20),269(100),225(2),209(2),151(27)	Eriodictyol hexoside	DAD/MS	0.15 ± 0.00
3	17.6	290	449	449(100),287(44),269(64),225(14),209(14),151(55)	Eriodictyol hexoside	DAD/MS	0.50 ± 0.01
4	19.6	356	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.47 ± 0.00
5	20.0	352	477	301(100)	Quercetin glucuronide	DAD/MS	0.24 ± 0.01
6	20.4	290	435	303(26),285(100)	Taxifolin pentoside	DAD/MS	0.83 ± 0.00
7	20.5	358	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.66 ± 0.01
8	20.9	352	463	301(100)	Quercetin hexoside	DAD/MS	0.78 ± 0.00
9	23.8	352	433	301(100)	Quercetin pentoside	DAD/MS	0.10 ± 0.01
10	24.2	346	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	0.02 ± 0.01
11	24.6	350	447	301(100)	Quercetin rhamnoside	DAD/MS	0.46 ± 0.01
12	35.4	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.09 ± 0.00
					Total		5.50 ± 0.05
				Rosa micrantha			
1	5.7	278	345	183(100),168(10)	Methyl gallate hexoside	DAD/MS	2.45 ± 0.02
2	16.0	294	435	303(54),285(100)	Taxifolin pentoside	DAD/MS	2.68 ± 0.01
3	17.1	296	449	449(100), 287(10),269(85),225(12),209(12),151(7)	Eriodictyol hexoside	DAD/MS	0.22 ± 0.01
4	17.8	290	449	449(100),287(44),269(66),225(12),209(25),151(44)	Eriodictyol hexoside	DAD/MS	0.63 ± 0.02
5	19.8	354	609	301(100)	Quercetin 3-*O*-rutinoside	Standard/DAD/MS	0.32 ± 0.00

6	20.3	358	477	301(100)	Quercetin glucuronide	DAD/MS	0.10 ± 0.01
7	20.6	292	435	303(53),285(100)	Taxifolin pentoside	DAD/MS	1.92 ± 0.00
8	20.7	352	463	301(100)	Quercetin 3-*O*-glucoside	Standard/DAD/MS	0.97 ± 0.01
9	21.2	352	463	301(100)	Quercetin hexoside	DAD/MS	0.90 ± 0.01
10	24.1	352	433	301(100)	Quercetin pentoside	DAD/MS	0.21 ± 0.00
11	24.4	352	623	315(100)	Isorhamnetin 3-*O*-rutinoside	Standard/DAD/MS	tr
12	25.1	346	447	301(100)	Quercetin rhamnoside	DAD/MS	0.71 ± 0.01
13	35.7	348	593	285(100)	Kaempferol rhamnosyl-hexoside	DAD/MS	0.05 ± 0.00
					Total		11.16 ± 0.04

tr-traces

Table 2. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of flavan-3-ols and galloyl derivatives in wild fruits.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (mg/100g)
				Arbutus unedo			
1	9.9	274	343	191 (100),169 (13)	Galloylquinic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	8.65 ± 0.00
2	11.3	278	331	169 (100)	Galloylhexoside acid	DAD/MS	1.02 ± 0.03
3	12.5	276	593	423(75),407(25),305 (100)	PA dimer (GC+C)[b]	DAD/MS	1.07 ± 0.00
4	12.9	276	325	169 (100)	Galloyl shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.61 ± 0.01
5	15.7	274	577	451(17),425(26),407(100), 289(78), 287(7)	B1 dimer	DAD/MS	8.56 ± 0.31
6	16.4	280	865	865(100),739(9), 713(9), 695(17), 577(9), 575(9), 425(12), 407(17), 289(12),287(12)	B-type procyanidin trimer	DAD/MS	4.11 ± 0.14
7	17.5	278	865	865(100),739(3), 713(3), 695(2), 577(11), 575(3), 425(4), 407(5), 289(9),287(7)	B-type procyanidin trimer	DAD/MS	2.76 ± 0.07
8	18.3	278	289	245 (70), 205 (33), 151 (21), 137 (33)	(+)-catechin	Standard/ DAD/MS	13.51 ± 0.93
9	18.4	278	495	343(42),191(100)	Digalloylquinic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.49 ± 0.42
10	19.4	278	1153	865 (7), 577 (8), 289 (100)	B-type procyanidin tetramer	DAD/MS	2.94 ± 0.07
11	22.4	278	577	451(17),425(75),407(100), 289(58), 287(17)	B-type procyanidin dimer	DAD/MS	3.35 ± 0.06
12	22.9	276	477	325(100),169(29)	Digalloylquinic shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	1.81 ± 0.04
13	23.9	270	633	633(100),463(12),301(70),275(5)	Strictinin ellagitannin	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	3.54 ± 0.01
14	28.3	270	477	325(100),169(54)	Digalloylquinic shikimic acid	Pawlowska et al., 2006/ Mendes et al., 2011 and Tavares et al., 2010/DAD/MS	2.51 ± 0.00
					Total flavan-3-ols		36.30 ± 0.70
					Total galloyl derivatives		24.63 ± 0.42
					Total		60.93 ± 0.27
				Rosa canina			

1	11.5	280	1351	1019(9),899(26),675(100),451(96),287(9)	PA trimer triglycoside	DAD/MS	0.77 ± 0.00
2	12.3	280	1189	1189(100), 898(23),739(17),575(10),449(17),289(7)	PA trimer diglycoside	DAD/MS	1.37 ± 0.00
3	12.9	280	819[a]	739(100),674(2),289(15)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	0.69 ± 0.04
4	13.5	274	819[a]	739(38),674(2),289(100)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	1.21 ± 0.07
5	14.9	280	739	449(18),425(29),407(100),289(47)	PA dimer monoglycoside	DAD/MS	3.27 ± 0.10
6	15.6	280	451	289(100)	(+)-catechin-hexoside	DAD/MS	1.62 ± 0.06
7	15.9	272	577	451(4),425(52),407(100),289(69)	Procyanidin dimer B1	DAD/MS	1.68 ± 0.09
8	16.3	278	577	425(31), 407(100), 289(25)	Procyanidin dimer B3	DAD/MS	1.50 ± 0.13
9	17.7	282	865	865(100),695(10), 577(50), 449(15), 407(25), 287(35)	B-type procyanidin trimer	DAD/MS	0.74 ± 0.08
10	17.9	280	739	739(100),449(11),425(11),407(48),289(22)	Procyanidin dimer monoglycoside	DAD/MS	1.32 ± 0.02
11	18.7	278	289	289(100),245(29), 205(8), 179(7), 137(4)	(+)-catechin	Standard/ DAD/MS	3.59 ± 0.17
12	30.1	280	865	865(100),739(14),577(21),575(21),425(14),407(14),289(21)	B-type procyanidin trimer	DAD/MS	2.14 ± 0.08
					Total flavan-3-ols		19.90 ± 0.51
				Rosa micrantha			
1	11.3	280	1351	1019(23),899(26),675(100),451(73),287(10)	PA trimer triglycoside	DAD/MS	2.32 ± 0.05
2	12.1	280	1189	898(37),739(19),575(14),449(22),289(100)	PA trimer diglycoside	DAD/MS	2.02 ± 0.04
3	12.7	280	819[a]	819(100),739(18),674(4),289(35)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	0.88 ± 0.07
4	13.2	280	819[a]	739(18),674(27),289(100)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	2.52 ± 0.06
5	13.8	280	1351	1019(13),899(13),675(50),451(100),287(14)	PA trimer triglycoside	DAD/MS	0.49 ± 0.01
6	14.5	280	739	739(100),449(27),425(13),407(63),289(43)	PA dimer monoglycoside	DAD/MS	4.93 ± 0.06
7	15.3	280	451	289(100)	(+)-catechin-hexoside	DAD/MS	2.64 ± 0.14
8	15.6	274	577	451(15),425(55),407(100),289(91)	Procyanidin dimer B1	DAD/MS	1.69 ± 0.04
9	17.6	280	739	739(100),449(25),425(15),407(47),289(22)	Procyanidin dimer monoglycoside	DAD/MS	0.94 ± 0.08
10	18.4	278	289	289(100),245(72), 205(32), 179(16), 137(18)	(+)-catechin	Standard/ DAD/MS	2.90 ± 0.06
11	19.8	284	819[a]	819(100),739(33),674(11),289(67)	PA tetramer triglycoside	Salminen et al., 2005/DAD/MS	1.54 ± 0.07
12	19.9	280	865	865(100),739(15),577(3),575(5),425(2),407(5),289(9)	B-type procyanidin trimer	DAD/MS	0.68 ± 0.12
13	21.6	280	851	851(100),739(6), 671(4), 561(62), 537(17), 407(10), 381(33), 357(6), 289(12)	PA trimer (2C+A)[b]	DAD/MS	1.80 ± 0.06
14	25.9	282	739	739(100),449(11),425(33),407(11),289(33)	PA dimer monoglycoside	DAD/MS	1.98 ± 0.11
15	26.8	280	851	851(100),739(6), 671(6), 561(71), 537(18),407(12),381(65), 357(12), 289(12)	PA trimer (2C+A)[b]	DAD/MS	1.40 ± 0.08
16	28.8	280	1027	1027(100),900(4),737(3),575(4),407(15),287(7)	PA trimer monoglycoside	DAD/MS	0.96 ± 0.34

17	29.9	282	865	865(100),739(6),577(25),575(31),425(13),407(25),289(25)	B-type procyanidin trimer	DAD/MS	1.63 ± 0.03
18	35.6	276	577	451(14),425(51),407(71),289(100)	B-type procyanidin dimer	DAD/MS	1.31 ± 0.03
					Total flavan-3-ols		32.62 ± 0.70

[a] $[M-H]^{2-}$; [b] C: (epi)catechin, A: (epi)afzelechin, GC: (epi)gallocatechin

Table 3. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of the anthocyanins of wild

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Identification type	Quantification (μg/100g dw)
				Arbutus unedo			
1	18.0	518	465	303(100)	Delphinidin 3-*O*-glucoside	Standard/DAD/MS	0.91 ± 0.01
2	22.4	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	11.40 ± 0.03
3	25.9	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.45 ± 0.02
					Total		13.77 ± 0.01
				Prunus spinosa			
1	23.1	518	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	19.83 ± 0.05
2	24.4	518	595	449(6), 287(100)	Cyanidin 3-*O*-rutinoside	Standard/DAD/MS	31.12 ± 0.11
3	30.9	518	463	301(100)	Peonidin 3-*O*-glucoside	Standard/DAD/MS	10.73 ± 0.16
4	31.4	520	609	463(3), 301(100)	Peonidin 3-*O*-rutinoside	Standard/DAD/MS	34.47 ± 0.03
5	32.2	518	419	287(100)	Cyanidin 3-*O*-pentoside	Standard/DAD/MS	1.49 ± 0.12
6	37.4	520	433	301(100)	Peonidin 3-*O*-pentoside	Standard/DAD/MS	0.26 ± 0.03
7	38.2	520	491	287(100)	Cyanidin 3-*O*-acetylglucoside	DAD/MS	1.77 ± 0.01
8	42.3	520	505	301(100)	Peonidin 3-*O*-acetylglucoside	DAD/MS	0.73 ± 0.05
					Total		100.40 ± 0.47
				Rosa canina			
1	22.6	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	0.68 ± 0.01
				Rosa micrantha			
1	22.5	516	449	287(100)	Cyanidin 3-*O*-glucoside	Standard/DAD/MS	1.19 ± 0.03

FIGURE LEGENDS

Figure 1. HPLC phenolic profiles of *Arbutus unedo* obtained at 370 nm (A), 280 nm (B) and 520 nm (C) used for recording flavone/ols, flavan-3-ols and galloyl derivatives, and anthocyanins, respectively. Note that different chromatographic conditions were used in each case.