Chemical characterization and bioactive properties of *Prunus avium* L.: The widely studied fruits and the unexplored stems

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Abstract

The aim of this study was to characterize sweet cherry regarding nutritional composition of the fruits, and individual phytochemicals and bioactive properties of fruits and stems. The chromatographic profiles in sugars, organic acids, fatty acids, tocopherols and phenolic compounds were established. All the preparations (extracts, infusions and decoctions) obtained using stems revealed higher antioxidant potential than the fruits extract, which is certainly related with its higher phenolic compounds (phenolic acids and flavonoids) concentration. The fruits extract was the only one showing antitumor potential, revealing selectivity against HCT-15 (colon carcinoma) (GI_{50}~74 μg/mL). This could be related with anthocyanins that were only found in fruits and not in stems. None of the preparations have shown hepatotoxicity against normal primary cells. Overall, this study reports innovative results regarding chemical and bioactive properties of sweet cherry stems, and confirmed the nutritional and antioxidant characteristics of their fruits.

Keywords: *Prunus avium*; Fruits/Stems; Nutrients; Phytochemicals; Antioxidant activity; Antitumor potential.

1. Introduction

Cherry is the common name for several species of Rosaceae family, Prunoideae subfamily, and *Prunus* genus that have their origin in the Asian continent, and produce fruits and hardwood. One of those species is *Prunus avium* L. (sweet cherry), being geographically distributed around the world, with greater prevalence in areas with a temperate climate, which encompasses much of Europe (Mediterranean and Central), north Africa, Near and Far East, South Australia and New Zealand, and temperate zones of the American continent (USA and Canada, Argentina and Chile) (Mariette, Tavaud, Arunyawat, Capdeville, Millan & Salin, 2010; Basanta, Plá, Raffo, Stortz & Rojas, 2014).

Sweet cherry is one of the most popular temperate fruits, being highly appreciated by consumers and studied by the scientific community due to its taste, color and sweetness, but also for its nutritional and bioactive properties (Usenik, Fabcic & Stampar, 2008; Serra, Seabra, Braga, Bronze, De Sousa & Duarte, 2010; Usenik, Fajt, Mikulic-Petkovsek, Slatnar, Stampar & Veberic, 2010; Liu et al., 2011; Serradilla, Lozano, Bernalte, Ayuso, López-Corrales & González-Gómez, 2011; Ballistreri, Continella, Gentile, Amenta, Fabroni & Rapisarda, 2013; Pacifico et al., 2014). The fruits present a moderate amount of carbohydrates, especially simple sugars (*e.g.*, glucose, fructose, sucrose and sorbitol), and organic acids (*e.g.*, malic, citric, succinic, lactic and oxalic acids) (Serrano, Guillen, Martinez-Romero, Castillo & Valero, 2005; Usenik et al., 2008; Usenik et al., 2010; Serradilla et al., 2011; Ballistreri et al., 2013; Pacifico et al., 2014). They have a low glycemic index (Brand-Miller & Foster-Powell, 1999), which is an advantage over other fruits and vegetables. Sweet cherry fruits are also considered a source of vitamins, especially vitamin C (Schmitz-Eiberger & Blanke, 2012) and minerals, such as potassium, phosphorus, calcium and magnesium (Yıgıt, Baydas, &

Güleryüz, 2009). Furthermore, they present high levels of water, reduced levels of fat, particularly saturated fat, being cholesterol-free and low in calories (McCune, Kubota, Stendell-Hollis & Thomson, 2011).

Sweet cherry fruits contain different phenolic compounds, including phenolic acids (hydroxycinnamic derivatives) and flavonoids (anthocyanins, flavan-3-ols and flavonols), that have been related with their antioxidant potential (Gao & Mazza, 1995, Gonçalves et al., 2004; Fazzari, Fukumoto, Mazza, Livrea, Tesoriere & Di Marco, 2008; Usenik et al., 2008; González-Gómez, Lozano, Fernández-León, Bernalte, Ayuso & Rodríguez, 2010; Serra et al., 2010; Usenik et al., 2010; Ballistreri et al., 2013; Pacifico et al., 2014).

Due to its high content in antioxidants, such as phenolic compounds and vitamins, *P. avium* beneficial effects have been recognized, namely in the prevention of cardiovascular diseases, cancer and other diseases related with oxidative stress (Beattie, Crozier & Duthie, 2005; Serra, Duarte, Bronze & Duarte, 2011a; Serra et al., 2011b). In recent years, the antitumor potential of *P. avium* fruit extracts have also been reported and related with phenolic compounds (Serra et al., 2010; Serra et al., 2011a and 2011b).

In opposition to the widely studied fruits and despite the traditional use of infusions and decoctions prepared from *P. avium* stems, as sedatives, diuretics and draining (Hooman, Mojab, Nickavar & Pouryousefi-Kermani, 2009; Di Cagno et al., 2011), little is known about their chemical composition and bioactive properties. Therefore, the aim of this study was to chemically characterize sweet cherry (*P. avium*) fruits and stems regarding individual hydrophilic and lipophilic compounds, and to evaluate their bioactive properties, namely antioxidant and antitumor potential.

2. Materials and methods

2.1. Plant material

Prunus avium L. fruits and stems were collected randomly, from growing plants in Bragança (Northern Portugal) in June 2013, and subsequently separated. The amount of samples collected from each part of *P. avium* was approximately around 600 g for fruits that gave 50 g of steams. The botanical identification was confirmed by the biologist, Dr. Carlos Aguiar of the Escola Superior Agrária of the Polytechnic Institute of Bragança (Trás-os-Montes, Portugal).

The samples were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh), mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

2.2. Standards and Reagents

Acetonitrile (99.9%), n-hexane (97%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), tocopherol and sugar standards. Phenolic compound standards were purchased from Extrasynthèse (Genay, France). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co.

(St Louis, MO USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Chemical characterization

2.3.1. Macronutrients composition

The fruits were analysed for their nutritional chemical composition (proteins, fat, carbohydrates and ash) through standard procedures (AOAC, 1995). The crude protein content (N × 6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Hydrophilic compounds

Free sugars. Free sugars were determined by a high performance liquid chromatograph (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smart line manager 5000) and an auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Stojković et al., 2013). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic). Quantification was based on the RI signal response of each standard, using the internal standard (IS, melezitose) method

and by using calibration curves obtained from the commercial standards of each compound. The results were expressed in g per 100 g of fresh weight.

Organic acids. Organic acids were determined following a procedure previously described by the authors (Dias et al., 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Coperation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 or 245 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of fresh weight.

2.3.3. Lipophilic compounds

Fatty acids. Fatty acids were determined after a transesterification procedure as described previously by the authors (Stojković et al., 2013). The fatty acids profile was analyzed with a DANI 1000 gas chromatographer (GC) equipped with a split/splitless injector and a flame ionization detector (FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Podohradska, Czech Republic) and expressed in relative percentage of each fatty acid.

Tocopherols. Tocopherols were determined following a procedure previously described by the authors (Stojković et al., 2013). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in µg per 100 g of fresh weight.

2.4. Evaluation of bioactive properties and phenolic compounds

2.4.1. Preparation of extracts, infusions and decoctions

The hydromethanolic extracts were obtained from the lyophilized stems and fruits. Each sample (1 g) was extracted by stirring with 30 mL of methanol/water (80:20, *v/v*) (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional 20 mL of methanol/water (80:20, *v/v*) (25 ºC at 150 rpm) for 1 h. The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove the methanol. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol followed and water; sugars and more polar substances were removed by passing through 10 mL of water and the purified samples were further eluted with 5 mL of methanol. The extract was concentrated under vacuum.

The infusions were prepared from the lyophilized stems. Each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusion was frozen and lyophilized.

The decoctions were also prepared from the lyophilized stems. Each sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained decoction was frozen and lyophilized.

The extracts, infusions and decoctions were redissolved in the corresponding solvent (final concentration 5 mg/mL) for antioxidant activity evaluation and phenolic compounds analysis, or water (final concentration 8 mg/mL) for antitumor activity evaluation.

The final solutions obtained were further diluted to different concentrations to be submitted to distinct bioactivity evaluation *in vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumor activity. Trolox and ellipticine were used as positive controls in antioxidant and antitumor activity evaluation assays, respectively (Dias et al., 2013).

2.4.2. In vitro antioxidant activity assays

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene

bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Dias et al., 2013; Roriz, Barros, Carvalho, Santos-Buelga & Ferreira, 2014).

2.4.3. Antitumor activity in human tumor cell lines

Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 ºC. Plates were then washed with deionised water and dried; sulphorhodamine B solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the

bound SRB was solubilised with 10 mM Tris (200 µL) and the absorbance was measured at 540 nm in the microplate reader mentioned above (Dias et al., 2013).

2.4.4. Hepatotoxicity

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Dias et al., 2013; Stojković et al., 2013).

2.4.5. Phenolic compounds composition

Phenolic compounds were determined by High-Performance Liquid Chromatography (HPLC, Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Dias et al., 2013; Roriz et al., 2014). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard

compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in mg per g of extract, infusion or decoction.

Anthocyanins. Each fruit sample (1 g) was extracted with 30 mL of methanol containing 0.5% trifluoroacetic acid (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 anthocyanins were further eluted with 5 mL of methanol:water (80:20, *v/v*) containing 0.1% TFA. The 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. Anthocyanins were determined by HPLC as previously described by the authors (Roriz et al., 2014). Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and in a MS connected to the HPLC system via the DAD cell outlet. The anthocyanins were tentatively identified by comparing their UV-vis and mass spectra with available standards and data in our compound library and the literature. The results were expressed in µg per g of extract, infusion or decoction.

2.5. Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. In the case of nutritional value, hydrophilic and lipophilic compounds, a Student´s *t*-test was used to determine the significant difference among two different samples, with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program.

3. Results and discussion

3.1. Chemical characterization of P. avium fruits and stems

The results of the nutritional characterization of *P. avium* (sweet cherry) fruits are shown in **Table 1**. Carbohydrates were the most abundant macronutrients, followed by proteins. Fat content was low, and the energetic contribution was ~58 kcal/100 g fw. Pacifico et al. (2014) reported the chemical composition of *P. avium*, being the values described by these authors very similar to the ones obtained in this study.

Hydrophilic compounds (free sugars and organic acids) were determined in fruits and stems. The main sugars and derivatives found either in fruits or stems were fructose, glucose and sorbitol (**Table 1**), being glucose the most abundant in fruits followed by fructose, while in stems all the compounds were found in similar amounts. Contrarily to the inexistent studies on stems, there are some reports on sugars composition of sweet cherry fruits (Usenik et al., 2008, 2010; Serradilla et al., 2011; Ballistreri et al., 2013; Pacifico et al., 2014). Those authors report the presence of the same sugars, with similar values, although some of them also detected the presence of sucrose (Usenik et al., 2008, 2010; Pacifico et al., 2014).

Oxalic, malic, ascorbic, citric and fumaric acids were found in the fruits of *P. avium* (**Table 1**), while stems presented oxalic, malic, shikimic and citric acids. Malic acid was the most abundant organic acid in both parts, also being reported by other authors in fruits as the main acid (Usenik et al., 2008, 2010; Serradilla et al., 2011; Ballistreri et al., 2013). Otherwise, those authors did not describe the presence of ascorbic acid, but identified shikimic acid. Schmitz-Eiberger and Blanke (2012) were the only authors reporting the presence of ascorbic acid in the fruits; nevertheless, the amount found by them was much higher than the one determined in this study, probably due to the different ripening stage of the analysed fruits, but these differences could also be explained by the different extraction methodologies applied.

Fatty acids (FA) and tocopherols composition of fruits and stems are shown in **Table 2**. In fruits, polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), whilst in stems SFA predominated over MUFA and PUFA. In fruits, the FA determined in higher percentages were linoleic acid (C18:2n6), oleic acid (C18:1n9), palmitic acid (C16:0) and α-linolenic acid (C18:3n3), while in stems the order of abundance was C16:0, C18:1n9, C18:2n6 and C18:3n3. Regarding tocopherols, δ-tocopherol was not detected in both parts of *P. avium*, being γ-tocopherol only present in steams; stems revealed the highest concentration in tocopherols. α-Tocopherol was the most abundant isoform in both parts, being more abundant in stems. To our best knowledge there are no reports on lipophilic compounds of *P. avium*.

3.2. Bioactivity of different preparations from P. avium fruits and stems

The *in vitro* antioxidant and antitumor properties of different preparations of *P. avium* fruits and stems were evaluated, and the results are given in **Table 3**. Due to the

traditional use *P. avium* stems, as sedatives, diuretics and draining (Hooman et al., 2009; Di Cagno et al., 2011), infusions and decoctions were also prepared and tested to compare with the bioactivity of the extracts obtained from stems and fruits.

The antioxidant potential of sweet cherry fruits was previously reported (Chaovanalikit & Wrolstad, 2004; Serra et al., 2010, 2011a, 2011b; Schmitz-Eiberger & Blanke, 2012). Nevertheless, in the present study, all the preparations obtained using stems revealed higher antioxidant potential than the tested extract from fruits. Particularly, stems extracts gave the highest antioxidant activity in all the assays, followed by decoctions and, then, infusions. This was probably related to the higher phenolic compounds concentration found in stems, in comparison with fruits (**Tables 4 and 5**). Regarding antitumor potential, no activity (up to 400 μg/mL) was observed for MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), HeLa (cervical carcinoma) and HepG2 (Hepatocellular carcinoma) cell lines. The fruits extract was the only one showing activity and revealed selectivity against HCT-15 (colon carcinoma) (GI_{50}~74 μg/mL; **Table 3**). This might be related to the presence of anthocyanins that were only found in fruits and not in stems (**Table 5**). In fact, the cytotoxicity of *P. avium* fruits for other human colon cancer cells (HT29) has been previously reported (Serra et al., 2010, 2011a, 2011b).

None of the tested preparations have shown hepatotoxicity against normal primary cells (**Table 3**; GI_{50} > 400 μg/mL for PLP2).

3.3. Analysis of phenolic compounds

The HPLC phenolic profiles of *P. avium* stems and fruits extract were recorded at 280 and 370 nm, and shown in **Figures 1A** and **B**. The peak characteristics and tentative identities are presented in **Tables 4** and **5**. Twenty-six compounds were detected in

stems, seven of which were phenolic acid derivatives and nineteen were flavonoids. Twelve compounds were identified in fruits (**Table 4**), three of which phenolic acid derivatives, three anthocyanins, and six other flavonoids.

Phenolic acids.

Sinapic acid (compound 7 in stems) was positively identified according to its retention, mass and UV-vis characteristics by comparison with a commercial standard. Compound 1 in stems and fruits ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid (neochlorogenic acid), yielding a base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >60% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford, Johnston, Knight and Kuhnert (2003) and Clifford, Knight and Kuhnert (2005). Compound 4 (stems) and compounds 3 and 4 (fruits) were identified as 3-*p*-coumaroylquinic acid, yielding the base peak at *m/z* 191, as reported by Clifford, Zheng and Kuhnert (2006). Furthermore, in fruits they were identified as *cis* and *trans* isomers of this compound; the assignment was made based on their relative order of elution, as hydroxycinnamoyl *cis* derivatives would be expected to elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory. Therefore, compound 4 (stems) and compound 3 (fruits) were identified as the *cis*-3-*p*-coumaroylquinic acid. Both 3-*O*-caffeoylquinic and 3-*p*-coumaroylquinic acids have been described in *P. avium* fruits (Gonçalves et al., 2004; Fazzari et al., 2008; González-Gómez et al., 2010; Usenik et al., 2008, 2010; Jakobek, Seruga, Voca, Sindrak & Dobricevic, 2009a; Jakobek, Seruga, Seruga, Novak & Medvidovic-Kosanovic, 2009b; Liu et al., 2011; Serra et al., 2010, 2011a and 2011b; Serradilla et al., 2011; Ballistreri et al., 2013; Pacifico et al., 2014).

In stems, compounds 2, 9 and 10 were identified as caffeic, *p*-coumaric and ferulic acid hexosides, based on the respective fragment ions released at *m/z* 179 [caffeic acid-H]$^-$,

163 [coumaric acid-H]$^-$ and 193 [ferulic acid-H]$^-$ after loss of a hexosyl moiety (-162 mu). Compound 5 presented the same pseudomolecular ion, UV spectra and fragmentation pattern to compound 2, and therefore it was tentatively identified as *trans* caffeic acid hexoside, taking into account the statement above.

Flavonoids

The following flavonoids detected in the stems were positively identified by comparison with commercial standards: catechin (compound 6), quercetin-3-*O*-rutinoside (compound 15), quercetin-3-*O*-glucoside (compound 16), kaempferol-3-*O*-rutinoside (compound 19), and kaempferol-3-*O*-glucoside (compound 21). The remaining compounds were tentatively assigned based on their UV and mass spectral characteristics, and previous identifications in *Prunus* species when available.

Compound 11 (in stems) and 7 (in fruits) presented a UV spectrum characteristic of flavonols (λ_{max} at 350 nm) and a pseudomolecular ion [M-H]$^-$ at *m/z* 771, releasing three fragments at *m/z* 609 ([M-162]$^-$, loss of a hexosyl moiety), 463 ([M-162]$^-$, loss of a deoxyhexoside moiety) and 301 [quercetin-H]$^-$ ([M-162]$^-$, loss of a hexosyl moiety). Thus, the compound was identified as a quercetin-*O*-deoxyhexosylhexoside-*O*-hexoside. Similarly, compound 12 in stems could be identified as a kaempferol-*O*-deoxyhexosylhexoside-*O*-hexoside. The deoxyhexosylhexoside substituent in these compounds could be associated to rutinose, owing to the positive identification of quercetin and kaempferol 3-*O*-rutinosides in *P. avium* stems. As far as we know, these compounds have not been previously identified in *P. avium*. Peak 20 with similar spectral characteristics as compound 15 (rutin) and a molecular mass 15 mu higher than it allowed assigning the compound as a methyl quercetin-*O*-rutinoside.

Tentative identifications of peaks 17 (genistein-7-*O*-glucoside), 18 (naringenin-7-*O*-glucoside, prunin) and 24 (chrysin-7-*O*-glucoside) were made taking into account their

previous description in the bark of *P. avium* and *P. cerasus* (Hasegawa, 1957; Geibel, Geiger & Treutter, 1990; Wang, Nair, Strasburg, Booren & Gray, 1999). Compound 8 in the fruit presented the same pseudomolecular ion ($[M-H]^-$ at *m/z* 433) as compound 18 in the stems, releasing a fragment at *m/z* 271 ($[M\text{-narigenin}]^-$). This peak eluted earlier than compound 18 in the stems, so that they cannot have the same identity, and therefore it was tentatively assigned as narigenin-*O*-hexoside.

Compounds 25 (stems) and 9 (fruits) presented a pseudomolecular ion $[M-H]^-$ at *m/z* 447 that would match with either sakuranin (sakuranetin-5-*O*-glucoside) or dihydrowogonin-7-*O*-glucoside. The presence of sakuranin in fruits of *P. avium* has been indicated by Treutter, Galensa, Feucht and Schmid (1987) and Serra et al. (2011b and 2010), whereas dihydrowogonin-7-*O*-glucoside was identified as a main component in callus, phloem and bark of *P. avium* (Treutter et al., 1985; Geibel et al., 1990). The fact that this peak was the majority compound in the stems of *P. avium* here analysed, as well as the observation that 7-*O*-glucosides are characteristic of *P. avium*, whereas 5-*O*-glucosides would be more typical of *P. cerasus* (Geibel et al, 1990; Geibel & Feucht, 1991), might support dihydrowogonin-7-*O*-glucoside rather than sakuranin as an identity for the compounds, although a definitive structure cannot be concluded. Compound 23 in the stems, with a pseudomolecular ion ($[M-H]^-$ at *m/z* 579) 132 mu higher than compound 25 and similar fragmentation pattern, could be assigned as a pentosylhexoside derivative of either dihydrowogonin or sakuranetin.

Compounds 3 (stems) and 6 (fruits) ($[M-H]^-$ at *m/z* 465) and compounds 8 and 13 (stems) ($[M-H]^-$ at *m/z* 449), all of them releasing a fragment ion from the loss of 162 mu (hexosyl moiety), may be assigned as *O*-hexosides of the dihydroflavonols taxifolin and aromadendrin, respectively, as those aglycones had been reported to occur in the wood of *P. avium* (Hasegawa, 1957). Although the nature and position of the sugar

cannot be established, compounds 3 and 8 (stems) might be speculated to be 7-*O*-glucosides, suggested to be characteristics of the bark of *P. avium* (Geibel et al., 1990; Geibel & Feucht, 1991). Compound 14, with similar characteristics as compound 8 and a molecular weight 15 mu higher than it, might correspond to a methyl aromadendrin-*O*-hexoside. Compound 5 ($[M-H]^-$ at *m/z* 611) in fruits, also released a fragment ion at *m/z* 303 ($[taxifolin-H]^-$; loss of a deoxyhexosylhexoside moiety, -308 mu), being tentatively identified as taxifolin-*O*-deoxyhexosylhexoside.

Compound 22 (stems) presented a pseudomolecular ion $[M-H]^-$ at *m/z* 549, releasing a fragment at *m/z* 255 ($[M-132-162]^-$) that might be associated to pinocembrin, a flavanone reported in the wood of different *Prunus* species (Hasegawa, 1957). Thus, the compound was tentatively assigned as pinocembrin-*O*-pentosyl-hexoside.

Finally, compound 26 in the stems presented a pseudomolecular ion $[M-H]^-$ at *m/z* 283 releasing a fragment ion at *m/z* 268 (loss of a CH_3 group), which might be coherent with a methyl genistein.

Anthocyanins. The anthocyanin profile obtained for *P. avium* fruit is shown in the chromatogram of **Figure 1C**, and the identities and concentrations of three identified anthocyanins are presented in **Table 5**. Cyanidin-3-*O*-glucoside (compound 10), cyanidin 3-*O*-rutinoside (compound 11) and peonidin-3-*O*-rutinoside (compound 12) were confirmed by comparison of their chromatographic, UV and mass spectral characteristics with data in our library. Cyanidin-3-*O*-rutinoside (compound 11) was the majority anthocyanin found in this sample. These three anthocyanins are the most commonly found in *P. avium* fruits (Gao & Mazza, 1995; Usenik et al., 2008; Usenik et al., 2010; Serradilla et al., 2011; Ballistreri et al., 2013).

In literature there are several reports on the identification and quantification of phenolic compounds in *P. avium* fruits, being the following compounds the most commonly found: phenolic acids (neochlorogenic, chlorogenic and *p*-coumaroylquinic acids), anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside, peonidin-3-*O*-rutinoside and pelargonidin-3-*O*-rutinoside), flavonols (rutin) and flavan-3-ols (catechin, epicatechin) (Gao & Mazza, 1995; Gonçalves et al., 2004; Fazzari et al., 2008; González-Gómez et al., 2010; Usenik et al., 2008; Usenik et al., 2010; Liu et al., 2011; Serra et al., 2010; Serradilla et al., 2011; Ballistreri et al., 2013; Pacifico et al., 2014). Moreover, Usenik et al. (2010) also reported the presence of some procyanidin derivatives, and Serra et al. (2011a) of quercetin-3-*O*-glucoside.

Overall, *P. avium* (sweet cherry) is one of the most popular temperate fruits, being highly appreciated by consumers and studied by the scientific community. In opposition to the widely studied fruits and despite the traditional medicinal use of infusions and decoctions prepared from *P. avium* stems, nothing is known about their chemical composition and bioactive properties. Therefore, the present study reports innovative results regarding chemical characterization and bioactive properties of sweet cherry stems. The traditional use of their infusions and decoctions was scientifically validated; otherwise, the extracts could be incorporated in nutraceutical or pharmaceutical products.

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Table 1. Characterization of *P. avium* fruits in macronutrients, and of fruits and stems in hydrophilic compounds.

Macronutrients in Fruits			
Moisture (g/100 g)	85.24 ± 2.52	Ash (g/100 g)	0.40 ± 0.10
Fat (g/100 g)	0.04 ± 0.00	Carbohydrates (g/100 g)	13.90 ± 1.72
Proteins (g/100 g)	0.42 ± 0.01	Energy (kcal/100 g)	57.65 ± 6.85
Hydrophilic compounds	Stems	Fruits	*t*-Students test *p*-value
Fructose	1.10 ± 0.05	5.47 ± 0.34	<0.001
Glucose	0.92 ± 0.05	6.02 ± 0.10	<0.001
Sorbitol	1.06 ± 0.05	1.62 ± 0.06	<0.001
Sum (g/100 g)	3.08 ± 0.06	13.85 ± 0.86	<0.001
Oxalic acid	64.97 ± 0.75	29.61 ± 0.95	<0.001
Malic acid	659.18 ± 0.68	715.78 ± 0.68	<0.001
Ascorbic acid	nd	1.92 ± 0.11	-
Shikimic acid	1.96 ± 0.04	nd	-
Citric acid	211.02 ± 0.62	6.53 ± 0.30	<0.001
Fumaric acid	nd	0.37 ± 0.02	-
Sum (mg/100 g)	937.13 ± 0.85	754.21 ± 1.23	<0.001

Results are expressed in fresh weight basis (Mean ± SD); nd- not detected.

Table 2. Characterization of *P. avium* stems and fruits in lipophilic compounds.

	Stems	Fruits	*t*-Students test *p*-value
C6:0	0.17 ± 0.00	0.07 ± 0.01	<0.001
C8:0	0.40 ± 0.00	0.12 ± 0.02	<0.001
C10:0	0.53 ± 0.03	0.17 ± 0.03	<0.001
C12:0	0.94 ± 0.02	0.70 ± 0.02	<0.001
C13:0	0.03 ± 0.00	tr	-
C14:0	1.86 ± 0.02	1.30 ± 0.01	<0.001
C14:1	0.00 ± 0.00	0.04 ± 0.00	<0.001
C15:0	0.75 ± 0.02	0.73 ± 0.01	0.212
C16:0	21.98 ± 0.40	22.27 ± 0.77	0.459
C16:1	0.82 ± 0.02	0.34 ± 0.07	<0.001
C17:0	1.20 ± 0.01	0.97 ± 0.02	<0.001
C18:0	8.39 ± 0.02	0.53 ± 0.04	<0.001
C18:1n9	18.61 ± 0.17	23.95 ± 0.54	<0.001
C18:2n6	17.64 ± 0.01	25.08 ± 0.07	<0.001
C18:3n3	16.83 ± 0.03	15.39 ± 0.20	<0.001
C20:0	2.95 ± 0.04	0.91 ± 0.02	<0.001
C20:1	0.11 ± 0.00	0.07 ± 0.01	<0.001
C20:3n3+C21:0	0.40 ± 0.03	0.14 ± 0.00	<0.001
C22:0	3.44 ± 0.20	0.64 ± 0.04	<0.001
C24:0	2.90 ± 0.10	0.58 ± 0.01	<0.001
SFA (percentage)	45.58 ± 0.18	35.00 ± 0.73	<0.001
MUFA (percentage)	19.55 ± 0.19	24.40 ± 0.46	<0.001
PUFA (percentage)	34.87 ± 0.01	40.60 ± 0.28	<0.001
α-tocopherol	512.58 ± 15.06	104.06 ± 9.39	<0.001
β-tocopherol	31.94 ± 4.30	11.81 ± 2.09	<0.001
γ-tocopherol	23.58 ± 1.08	nd	-
Sum (µg/100 g)	568.10 ± 18.28	115.87 ± 11.48	<0.001

The results of fatty acids are expressed in relative percentage; The results of tocopherols are expressed in fresh weight basis (mean ± SD); tr- traces. Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1c); *cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); Behenic acid (C22:0); Lignoceric acid (C24:0).

Table 3. Bioactive properties of different preparations from *P. avium* fruits and stems.

Samples	Stems			Fruits
	Extracts	Infusions	Decoctions	Extracts
Antioxidant activity (EC_{50}, mg/mL)				
DPPH scavenging activity	0.36 ± 0.01^{d}	0.63 ± 0.01^{c}	0.54 ± 0.01^{b}	0.99 ± 0.01^{a}
Reducing power	0.18 ± 0.02^{d}	0.44 ± 0.03^{b}	0.31 ± 0.01^{c}	0.57 ± 0.01^{a}
β - Carotene bleaching inhibition	0.30 ± 0.01^{d}	0.42 ± 0.06^{b}	0.35 ± 0.04^{c}	1.80 ± 0.04^{a}
TBARS inhibition	0.07 ± 0.00^{d}	0.24 ± 0.01^{b}	0.13 ± 0.01^{c}	1.46 ± 0.09^{a}
Antitumor activity (GI_{50} values, µg/mL)				
HCT-15 (colon carcinoma)	>400	>400	>400	73.51±6.37
Hepatotoxicity (GI_{50} value, µg/mL)				
PLP2	>400	>400	>400	>400

The antioxidant activity was expressed as EC_{50} values (Mean ± SD), what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 µg/mL (reducing power), 42 µg/mL (DPPH scavenging activity), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). GI_{50} values (Mean ± SD) correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.42 µg/mL (HCT-15) and 2.06 µg/mL (PLP2). In each row different letters mean significant differences between species ($p<0.05$).

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in *P. avium* stem extracts, infusions and decoctions.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)		
						Extracts	Infusions	Decoctions
1	5.2	328	353	191(100),179(60),173(5),135(50)	3-*O*-Caffeolyquinic acid	0.43 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
2	6.4	326	341	179(100)	*cis* Caffeic acid hexoside	0.17 ± 0.01	0.10 ± 0.01	0.12 ± 0.02
3	6.6	282,342sh	465	303(100)	Taxifolin-7-*O*-hexoside	0.79 ± 0.04	0.29 ± 0.01	0.19 ±0.01
4	6.9	312	337	191(21),173(6),163(100),155(54)	*p*-coumaroylquinic acid	0.53 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
5	7.1	322	341	179(100)	*trans* Caffeic acid hexoside	0.49 ± 0.02	0.07 ± 0.01	0.09 ± 0.01
6	7.8	278	289	245(56), 203(19), 137(44)	Catechin	3.74 ± 0.01	0.42 ± 0.04	0.44 ± 0.05
7	9.4	312	223	-	Sinapic acid	0.29 ± 0.01	0.17 ± 0.01	0.15 ± 0.01
8	9.6	285,342sh	449	287(100)	Aromadendrin-7-*O*-hexoside	2.66 ± 0.07	1.22 ± 0.04	0.86 ± 0.02
9	10.9	312	325	163(100)	*p*-coumaric acid hexoside	0.68 ± 0.04	0.32 ± 0.02	0.25 ± 0.01
10	13.1	324	355	193(100)	Ferulic acid hexoside	0.30 ± 0.03	0.20 ± 0.01	0.17± 0.01
11	15.4	350	771	609(100),463(20),301(41)	Quercetin-*O*-rutinoside-*O*-hexoside	0.44 ± 0.01	0.02 ± 0.00	0.03 ± 0.00
12	15.6	346	755	593(100),447(47),285(55)	Kaempferol-*O*-rutinoside-*O*-hexoside	0.55 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
13	17.5	286,334sh	449	287(100)	Aromadendrin-*O*-hexoside	0.31 ± 0.04	tr	tr
14	19.3	288,348sh	463	301(100),286(17)	Methyl-aromadendrin-*O*-hexoside	0.06 ± 0.01	tr	tr
15	19.7	356	609	301(100)	Quercetin-3-*O*-rutinoside	0.87 ± 0.02	nd	nd
16	21.0	356	463	301(100)	Quercetin-3-*O*-glucoside	0.27 ± 0.02	nd	nd

20	24.4	356	623	315(75),300(21)	Methyl quercetin-*O*-rutinoside	0.15 ± 0.01	nd	nd
21	24.8	352	447	285(100)	Kaempferol-3-*O*-glucoside	0.30 ± 0.01	nd	nd
22	29.4	286,336sh	549	255(100)	Pinocembrin-*O*-pentosylhexoside	0.23 ± 0.01	0.05 ± 0.01	0.03 ± 0.00
23	30.1	290,340sh	579	285(90),270(14)	Dihydrowogonin/sakuranetin-*O*-pentosylhexoside	0.36 ± 0.01	tr	tr
24	32.2	258/316	415	253(100)	Chrysin-7-*O*-glucoside	0.50 ± 0.01	tr	tr
25	33.4	286/346	447	285(85),270(100)	Dihydrowogonin 7-*O*-glucoside/sakuranetin 5-*O*-glucoside	13.63 ± 0.05	8.49 ± 0.13	5.66 ± 0.05
26	34.3	272/348	283	268(100)	Methyl genistein	0.31 ± 0.01	0.08 ± 0.02	0.03 ± 0.00
					Phenolic acids	2.90 ± 0.07[a]	1.04 ± 0.04[b]	0.98 ± 0.07[c]
					Flavonoids	29.54 ± 0.10[a]	10.83 ± 0.27[b]	7.39 ± 0.12[c]
					Total phenolic compounds	32.44 ± 0.17[a]	11.88 ± 0.30[b]	8.37 ± 0.19[c]

nd-not detected; tr-traces. In each row, different letters mean significant differences (p<0.05).

Table 5. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in *P. avium* fruit extracts.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Identification	Quantification (mg/g)
1	5.2	328	353	191(100),179(66),173(9),161(10),135(62)	3-*O*-caffeoylquinic acid	0.83 ± 0.03
2	5.7	278	451	289(20),245(5),137(100)	Catechin hexoside	1.68 ± 0.01
3	6.9	312	337	191(68),173(8),163(100),155(3),119(59)	*cis p*-coumaroylquinic acid	0.56 ± 0.01
4	7.1	310	337	191(51),173(8),163(100),155(5),119(40)	*trans p*-coumaroylquinic acid	0.23 ± 0.02
5	7.9	342	611	303(13),285(76)	Taxifolin-*O*-deoxyhexosylhexoside	0.66 ± 0.01
6	8.5	350	465	303(22),285(100)	Taxifolin-*O*-hexoside	0.13 ± 0.01
7	15.3	350	771	609(100),463(25),301(42)	Quercetin-*O*-rutinoside-*O*-hexoside	0.42 ± 0.01
8	15.6	268,sh342	433	271(20),253(75)	Narigenin-*O*-hexoside	0.17 ± 0.01
9	33.4	288,sh346	447	285(92),270(22)	Dihydrowogonin 7-*O*-glucoside/sakuranetin 5-*O*-glucoside	0.62 ± 0.01
					Phenolic acids	1.62 ± 0.05
					Flavonoids (non-anthocyanins)	3.96 ± 0.04
					Total phenolic compounds	5.58 ± 0.09
Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Identification	Quantification (µg/g)
10	18.2	512	449	287(100)	Cyanidin-3-*O*-glucoside	2.19 ± 0.27
11	19.6	518	595	449(10),287(100)	Cyanidin-3-*O*-rutinoside	14.50 ± 0.64
12	25.5	524	609	463(8),301(100)	Peonidin-3-*O*-rutinoside	0.64 ± 0.01
					Anthocyanins	17.34 ± 0.91