ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 2/2017
265–271
https://doi.org/10.18388/abp.2016_1374

Regular paper

Analysis of glycosylated flavonoids extracted from sweet-cherry stems, as antibacterial agents against pathogenic *Escherichia coli* isolates

Alfredo Aires[1]✉, Carla Dias[2], Rosa Carvalho[3] and Maria José Saavedra[2]

[1]Centre for the Research and Technology for Agro-Environment and Biological Sciences, CITAB, Universidade de Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, Portugal; [2]Animal and Veterinary research Centre, CECAV, Universidade de Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, Portugal; [3]Agronomy Department, Universidade de Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, Portugal

The aim of this study was to evaluate the bioactivity of flavonoids extracted from sweet-cherry stems which are often used by a traditional system of medicine to treat gastro-intestinal and urinary tract infections but lacking any consistent scientific evidence; moreover the information about the class of phenolics, their content and the potential bioactivity of such material is very scarce. Thus, in this context, we have set a research study in which we evaluated the profile and content of phenolics extracted from sweet-cherry stems through a conventional (70ºC and 20 min) and ultrasound assisted extraction (40 kHz, room temperature and 20 min). The extracts were phytochemically characterized by using an HPLC-DAD-UV/VIS system and assayed by an *in vitro* minimum inhibitory concentration (MIC) bioassay against *Escherichia coli* isolates. Simultaneously, the total antioxidant activities were measured using the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\cdot+}$) radical cation assay. Our results indicate that sweet-cherry stems have a high content of sakuranetin, ferulic acid, *p*-coumaric acid, *p*-coumaroylquinic acid, chlorogenic acid and its isomer neochlorogenic acid. Their average levels were highly affected by the extraction method used ($p<0.001$). The same trend was observed for total antioxidant activity and MIC values. The extracts produced with ultrasounds presented both, a higher total antioxidant activity and a lower minimum inhibitory concentration. Statistical analyses of our results showed a significant correlation ($p<0.01$) of total antioxidant activity and minimum inhibitory concentration with phenolics present in the extracts studied. Thus, we can conclude that cherry stems can be further exploited to purify compounds and produce coproducts with enhanced biologically added value for pharmaceutical industry.

Key words: flavonoids, agro-food wastes, enhanced extraction, antimicrobial agents, pathogenic bacteria

Received: 15 June, 2016; revised: 08 August, 2016; accepted: 13 February, 2017; available on-line: 14 April, 2017

✉e-mail: alfredoa@utad.pt

Abbreviations: $ABTS^{\cdot+}$, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonat; ANOVA, two-way analysis of variance; IC50, inhibition concentration at 50% inhibition; MIC, minimum inhibitory concentration; SPSS, statistical Package for the Social Sciences; TAA, total antioxidant activity; UAE, ultrasound assisted extraction; S.D., standard deviation.

INTRODUCTION

In the last decades, several studies have been published about the protective effect of phytochemicals from fruits, vegetables and medicinal plants or even some plant residues against important inflammatory and neurodegenerative diseases (Bansode *et al*., 2012; Sánchez-Vioque *et al*., 2013).This protection is often ascribed to the content of vitamins, minerals, oils, and secondary metabolites such as phenolic acids, flavonoids and anthocyanins. The richness and diversity of polyphenols in foods and crops have been two of the major reasons for looking to foods and their wastes as the source of natural phytochemicals with anti-inflammatory, antimicrobial and neuroprotective effects (Lobo *et al*., 2012). Every year, several tons of different types of food wastes are generated by the industry, without any substantial usage. Among them, fruit peduncles, leaves, stems, peels, barks and seeds are largely neglected, but according to the literature the content of bioactive compounds in these parts, when compared to the edible counterparts, can be sometimes higher (Fernando *et al*., 2013). In a recent review (Ajil *et al*., 2012), it was reported that food industry wastes can reach nearly 30% of the worldwide agricultural production, and those materials can comprise several nutraceutical compounds, such as polyphenols, carotenoids and dietary fibers. The same trend is observed in Portugal, a traditional producer of fruits and vegetables, in which every year tons of wastes from crops, including cherry (peduncles), are produced without any substantial use, despite their traditional use in folk medicine in infusions and decoctions used as diuretic and anti-inflammatory beverages (Sargin *et al*., 2013). Although the chemical composition of sweet-cherry is well established (Ballisteri *et al*., 2013; Alfieri *et al*., 2104), limited information is available about the phytochemical composition and bioactivities of their wastes.

Another important issue related to the use of natural compounds has been the search for new and effective compounds against recurrent bacterial infections, particularly those associated with the antibiotic resistance phenomena. Although new generations of antibiotics were produced by pharmacological industries (Coates *et al.* 2011), several antibiotics have failed to discourage the growth of many bacteria, including *Escherichia coli*, one of the most prevalent Gram-negative pathogen causing several diseases in humans and animals, particularly in poor communities (Da Silva *et al*., 2012). Although antimicrobial therapy is a significant tool in the treatment of these infections, their widespread resistance has become a great concern in the human and veterinary medicine (Schoevaerdts *et al*., 2011). Therefore, in the recent years we have assisted in an increment of research studies about

the use of natural compounds, extracted from plants, against important pathogenic human and animal agents. In fact, plants have an almost infinite ability to produce compounds with different bioactive purposes that the humans and animals cannot synthesize. Therefore, they can be very useful for finding new, simple or complex compounds with antimicrobial activity. Thus, the aim of this study was to evaluate the phytochemical potential and the biological value of cherry wastes in order to extract effective phytochemicals against *E. coli* isolates. Additionally, the goal was to assess if these by-products can withstand the industrial processes of extraction used for production of coproducts with a highly added value for supplements or pharmaceutical applications.

MATERIALS AND METHODS

General experimental procedure. To develop this work, 1 kilogram of plant wastes from sweet-cherry (*Prunus avium* L.), was directly collected from a Portuguese private company from the food industry transformation sector. The sweet-cherry wastes were collected and separated into leaves and stems. The leaves were discharged and the stems were used in this study. The stems were then dehydrated in a freeze-dry system (UltraDrySystems™, USA), milled and reduced to a fine powder, and stored in dark flasks at 4ºC in a dark environment until chemical extraction. For extraction of phytochemicals, two different methods were performed in order to establish a simple, practical and feasible extraction procedure: 1) a conventional extraction and 2) an ultrasound assisted extraction (UAE).

Extraction. In the conventional extraction, 100 mg of dry powder was added to a screw cap tube (10 mL) and mixed with 10 mL of the extraction solution (70% methanol). Next, each suspension was agitated by vortexing (Genie 2, Fisher Scientific, UK) and heated at 70°C (1083, GFL-Gesells chaft ffur Labortechnik mbH, Germany) for 20 minutes, with agitation taking place every 5 minutes. Then, the mixtures were centrifuged at 4000 rpm for 5 min (Kubota, Japan), filtered (Whatman No. 1 paper) and completely dried in a rotary evaporator under vacuum. The residues were then re-suspended in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Tauferkichen, Germany) to obtain a final concentration of 5 $mg \times mL^{-1}$. This final suspension was stored at –20°C until subsequent analysis. In the UAE extraction, the same amount of dry powder of each sample that was used in the conventional method was weighed into 10 mL screw cap tubes, mixed with 10 mL of the extraction solution (70% methanol), and placed in an ultrasonic bath device (Bandelin Electronics, Berlin, Germany) (34 cm × 10 cm × 31 cm), with 40 kHz transducers annealed to the bottom. The ultrasonic device was turned on for the pre-set extraction time (20 minutes) at room temperature (20 ± 0.1°C). Then, the extracts were centrifuged at 4000 rpm for 15 min (Kubota, Japan), the supernatants were collected, filtered (Whatman No.1 paper) and then submitted to the same final procedure as used for the conventional extraction.

Phytochemical analysis. The evaluation of phytochemical composition of each extract was performed by HPLC – DAD-UV/VIS system with 1% trifluoroacetic acid (TFA) (solvent A) and acetonitrile with 1% TFA (solvent B) as eluents. The elution was per-

Table 1. Phenolic compounds and respective retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}) of the studied by-product extracts (by order of elution).

Phenolic compound identified	Rt (min)	UV (nm)	UV-DAD/VIS bands (nm)	Class
Neochlorogenic acid	14.89	320	244, 300sh, 326	Hydroxycinnamic acid
(+)-Catechin	17.23	280	231, 280	Flavan-3-ols
Cholorogenic acid	17.39	320	243,300sh, 326	Hydroxycinnamic acid
p-Coumaric acid	18.81	320	230, 277sh, 311	Hydroxycinnamic acid
(-)-Epicatechin	19.60	280	232, 279	Flavan-3-ols
Quercetin-3-*O*-rutinoside	21.60	370	256, 266sh, 355	Flavonol
Kaempferol-3-*O*-rutinoside	22.79	370	266, 329sh, 369	Flavonol
Ferulic acid	23.13	320	244, 296sh, 328	Hydroxycinnamic acid
Hydroxycynnamic acid derivative	25.40	320	253, 288, 296	Hydroxycinnamic acid
p-Coumaroylquinic acid	26.54	320	261, 312	Hydroxycinnamic acid
Apigenin	26.89	370	230, 268sh, 338	Flavonol
Sakuranetin	27.93	280	229, 292sh, 340	Flavanone
Isosakuranetin	34.47	280	230, 288sh, 340	Flavanone

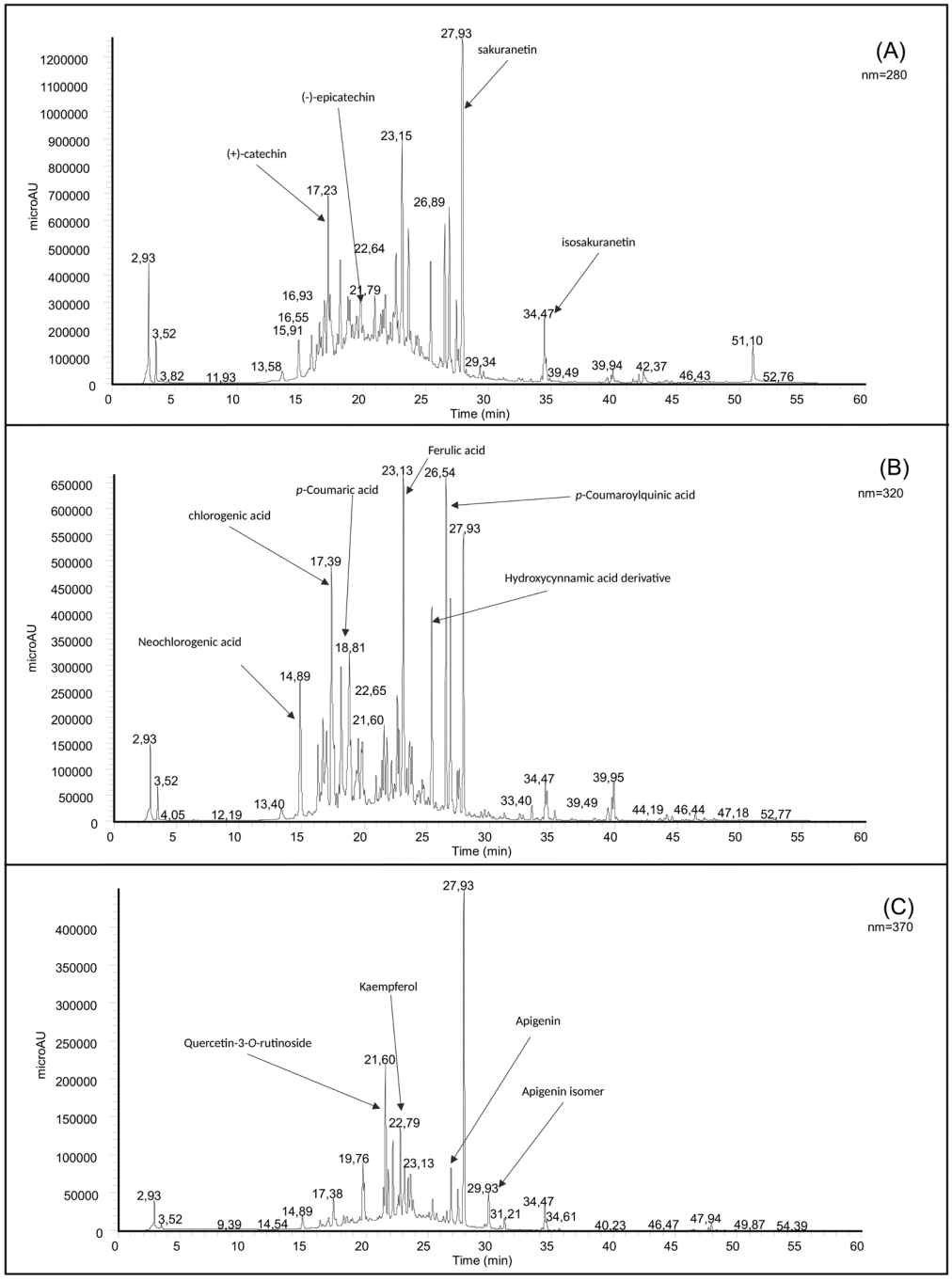

Figure 1. Chromatograms of sweet-cherry wastes (stems), recorded at different wavelengths: 280 nm (A), 320 nm (B) and 370 nm (C).

formed at a flow rate of 1 mL min^{-1} of solvent, with the gradient starting with 100% of water, and the injection volume of 10 µL. Chromatograms were recorded at 280, 320, 370 and 520 nm with a C18 column (250 × 46 mm, 5 µm). Phytochemicals were identified using peak retention time, UV spectra and UV max absorbance bands, and compared with those found for external commercial standards and in the literature. The quantification was made using internal (naringin, Sigma-Aldrich, Germany), and external standards. The external standards (–)-epicatechin, (+)-Catechin, apigenin, chlorogenic acid, ferulic acid, kaempferol-3-*O*-rutinoside, isosakuranetin, neochlorogenic acid, *p*-coumaric acid, quercetin-3-*O*-rutinoside and sakuranetin, were purchased from Extrasynthese (Cedex, France). All standards were freshly prepared in 70% methanol (methanol:water) at 1 mg mL^{-1}. Methanol and acetonitrile were purchased from Panreac chemistry (Lisbon, Portugal) and Sigma-Aldrich (Taufkirchen, Germany), respectively. The aqueous solutions were prepared using ultra-pure water (Milli-Q, Millipore).

Total antioxidant activity (TAA) *in vitro* bioassay. Simultaneously with the phytochemical determination, the TAA was measured by an *in vitro* 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\bullet+}$) radical cation assay and was determined in a 96-well microplate. ABTS was generated by mixing a 7 mM ABTS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154

Table 2. Average levels of individual polyphenols identified in the cherry wastes, expressed as μg g^{-1} dw

Phenolics	Conventional	UAE	*p-Value*[2]
Neochlorogenic acid	145.3±0.4	167.2±0.4	$p<0.001$
(+)-Catechin	159.1±0.4	225.4±0.6	$p<0.001$
Cholorogenic acid	167.4±0.4	193.0±0.5	$p<0.001$
p-Coumaric acid	77.3±0.2	92.9±0.2	$p<0.001$
(-)-Epicatechin	87.3±0.2	110.2±0.3	$p<0.001$
Quercetin-3-O-rutinoside	66.6±0.2	68.9±0.2	$p<0.001$
Kaempferol-3-O-rutinoside	38.9±0.1	39.7±0.1	$p<0.01$
Ferulic acid	261.3±0.7	278.7±0.7	$p<0.001$
Hydroxycynnamic acid derivative	130.3±0.3	159.9±0.4	$p<0.001$
p-Coumaroylquinic acid	209.0±0.5	251.0±0.6	$p<0.001$
Apigenin	32.7±0.1	52.5±0.1	$p<0.001$
Sakuranetin	644.1±1.6	737.6±1.8	$p<0.001$

[1]Values expressed as mean ±S.D. of three replicates. [2]Probability test values obtained by ANOVA variance analysis.

mM NaCl) with 2.5 mM potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm when using a U-2000 spectrophotometer (serial 121-0120, Hitachi Ltd., Tokyo, Japan). For each sample, several dilutions (100 μL) in decreasing concentrations starting from 5 mg × mL^{-1}, were allowed to react with a fresh ABTS solution (900 μL), and then the absorbance was measured 6 min after initial mixing. The TAA was expressed as follows: TAA (%) = [((solvent absorbance – sample absorbance)/solvent absorbance) ×100]. All determinations were performed in triplicate. The half maximal inhibitory concentration (IC_{50}) was determined in order to compare the TAA, and lower IC_{50} means better radical scavenging activity.

***In vitro* antimicrobial activity bioassay**. After the determination of TAA, an *in vitro* bioassay of antimicrobial activity of extracts against bacterial pathogenic isolates was performed. For that, Gram-negative isolates of *Escherichia coli* collected from gastrointestinal tract segments, isolated and purified at the Microbiology laboratory of University of Trás-os-Montes e Alto Douro, were used. The identifications were performed by means of standard biochemical classification techniques (Murray *et al.* 1999), using API 20E, API 20NE, API Staphy and API Step (BioMerieux), according to the procedure described previously (Joergensen *et al.*, 2009), followed by genetic identification through 16S rRNA sequencing. After this identification, a minimum inhibitory concentration (MIC) assay (Simões *et al.*, 2009) in 96-well microplates was performed. It is widely accepted that phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that yield minimum inhibitory concentration (MIC) in the range of 100–1000 μg mL^{-1} (Sarker *et al.*, 2007). Thus, we prepared different extracts with a maximum concentration of 1000 μg of total phenolics in 10% DMSO. Next, 100 μL of each extract (1000 μg of total phenolics in 10% DMSO) and 1000 μg of a standard antibiotic were added into the first row of the 96 well microplates. Then, two fold serial dilutions were performed in additional wells containing 100 μL of nutrient broth. Then, bacterial suspensions (20 μL) were added to each well to obtain a concentration of 5×10^5 cfu mL^{-1} and 20 μL of the resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 mL of sterile double-distilled water) were finally added into each well. Positive and negative controls were included: a column with a ciprofloxacin (Oxoid, England) served as a positive control, and three negative controls were used (a column without any bioactive compounds, a column without the bacterial solution (20 μL of nutrient broth was used instead) and a column with a DMSO solution). The plates were then incubated at 37°C for 24 h, and the color changes were assessed visually. Bacterial growth was indicated by color modification from purple to pink (or colorless) and the MIC was defined as the lowest concentration of the tested extract or antibiotic that completely inhibited the visible bacterial growth. This assay was done in triplicate. To classify the antimicrobial activity effect, the following criteria were established (Kuete *et al.*, 2011): very high, if MIC values were below 100 μg mL^{-1}; high, when 100<MIC<625 μg mL^{-1}; moderate, when 625<MIC≤1000 μg mL^{-1}; and null, if MIC>1000 μg mL^{-1}. All determinations were performed in triplicate.

Table 3. Antimicrobial properties of sweet-cherry wastes based on minimum inhibitory concentration (MIC)

Extraction method	Bacterial isolates	MIC (mg mL^{-1} of total polyphenols)	Antibacterial effect[1]
Conventional extraction	*E. coli* MJS 260	0.250	High
	E. coli MJS 294	0.250	High
	E. coli JH270	0.125	High
UA extraction	*E.coli* MJS260	0.250	High
	E. coli MJS 294	0.125	High
	E. coli JH270	0.125	High

[1]Antimicrobial activity is considered: very high, if MIC<100 μg×mL^{-1}; high, when 100<MIC≤500 μg×mL^{-1}; moderate, when 500<MIC<1000 μg×mL^{-1}; weak, when MIC=1000 μg×mL^{-1}, and null, if MIC>1000 μg×mL^{-1}.

Statistical analysis. All data were expressed as mean values ± standard deviation (S.D.). The results were analyzed using one-way ANOVA, followed by the Duncan multiple range test, based on

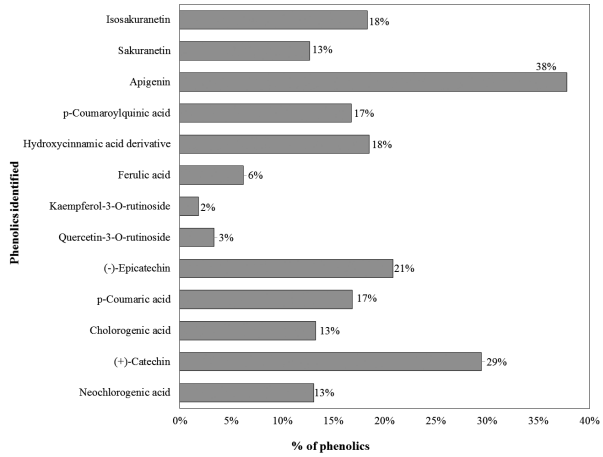

Figure 2. Increment in phenolic content when UAE method is used, when compared to a conventional method, expressed as percentage (%).

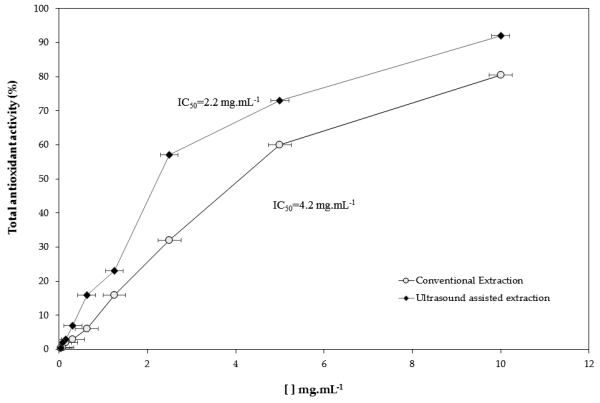

Figure 3. Graphical representation of total antioxidant activity (TAA) determined at different extract concentration. IC_{50} values are included in the graph.

confidence level equal or higher than 95% ($p<0.05$). A Pearson's correlation was performed to establish a clear trend between bioactivities (TAA and MIC) and the levels of phytochemicals. The SPSS v.17 software (SPSS-IBM, Orchard Road-Armonk, New York, NY, USA) was used to carry out this analysis.

RESULTS AND DISCUSSION

The results of the polyphenol profile determined by HPLC are presented in Table 1 and Fig. 1. The average content of each polyphenol determined in this study is presented in Table 2. The analysis revealed different types of polyphenols grouped into flavanones, flavan-3-ols and hydroxycinnamic acid types (Table 1), and higher levels were found for sakuranetin + isosakuranetin (on average, 35% of total polyphenols identified), neochlorogenic acid and chlorogenic acid (14.5% of total polyphenols identified), *p*-coumaric acid + *p*-coumaroylquinic acid (13.6% of total polyphenols identified), ferulic acid (11.6% of total polyphenols identified) and + catechin + epicatechin (12.5% of total polyphenols identified) (Table 2). The highest levels for each polyphenol were consistently obtained for the extracts produced with the UAE method when compared to those obtained by a conventional method ($p<0.001$).

Table 4. Pearson´s coefficient correlations[1,2]

	IC_{50} values	Minimum inhibitory concentration (MIC)
Neochlorogenic acid	–0.9996**	–0.9996**
	($p<0.001$)	($p<0.001$)
(+)-Catechin	–0.9999**	–0.9999**
	($p<0.001$)	($p<0.001$)
Cholorogenic acid	–0.9996**	–0.9996**
	($p<0.001$)	($p<0.001$)
p-Coumaric acid	–0.9998**	–0.9998**
	($p<0.001$)	($p<0.001$)
(–)-Epicatechin	–0.9998**	–0.9998**
	($p<0.001$)	($p<0.001$)
Quercetin-3-O-rutinoside	–0.9929**	–0.9729**
	($p<0.001$)	($p<0.001$)
Kaempferol-3-O-rutinoside	–0.9730**	–0.9760**
	($p<0.001$)	($p<0.001$)
Ferulic acid	–0.9780**	–0.9880**
	($p<0.001$)	($p<0.001$)
Hydroxycynnamic acid derivative	–0.9998**	–0.9998**
	($p<0.001$)	($p<0.001$)
p-Coumaroylquinic acid	–0.9997**	–0.9997**
	($p<0.001$)	($p<0.001$)
Apigenin	–0.9976**	–0.9983**
	($p<0.001$)	($p<0.001$)
Sakuranetin	–0.9995**	–0.9995**
	($p<0.001$)	($p<0.001$)
Isosakuranetin	–0.9998**	–0.9997**
	($p<0.001$)	($p<0.001$)

[1]**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level. [2]In brackets, the respective *p* value is given.

The general tendency observed was increment in phenolic contents when the UAE method was used. The average levels of phenolic increments, obtained with UAE when compared to the conventional method, varied between 2% and 38% (Fig. 2), and the apigenin, catechin, epicatechin, sakuranetin and isosakuranetin flavonoids, were the flavonoids with the highest percentage of increment (Fig. 2). This trend is in agreement with previous studies (Schneider *et al.* 2006; Ashokkumar *et al.*, 2008; Tiwari *et al.*, 2010) where an increment in the phenolic contents was found, particularly of the flavonoid types, when UAE was used to extract them. The increment of pressure during sonication provokes the decomposition of radicals and the hydroxylation of aromatic rings into ortho-, meta- and

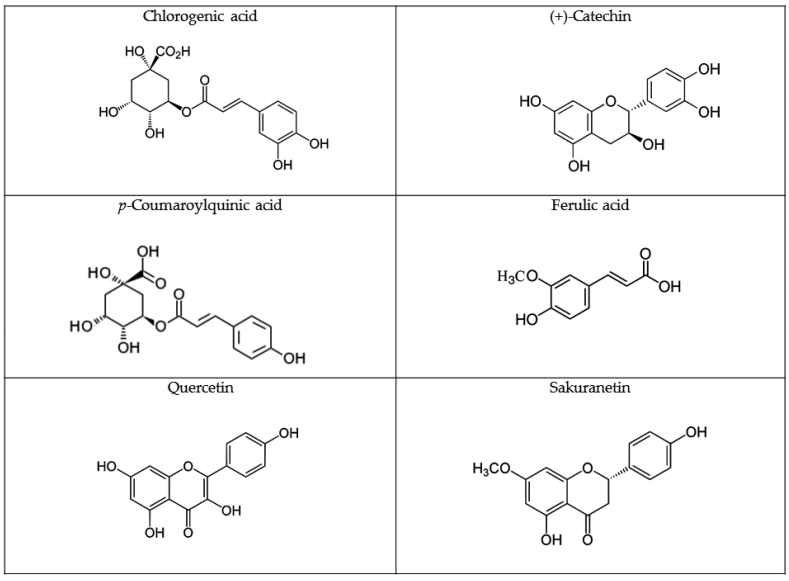

Figure 4. Chemical parent structures of the phenolics identified in the current study.

para-positions, increasing their solubility and movement from cells towards the solvents (Ashokkumar *et al.*, 2008; Tiwari *et al.*, 2010), and thus incrementing their concentration in extracted solutes. However, the amplitude of this effect is dependent on intrinsic properties of the matrices and extraction factors, such as the temperature, pH, light, and time (Alihourchoi *et al.*, 2013). Based on our results, the UAE seems to be very effective since it increases extraction of polyphenols from the flavonoid class. A similar trend was found in the TAA (Fig. 3) and the antimicrobial activity bioassays (Table 3). In these experiments, the extracts produced with UAE yielded the lowest values of IC_{50} (Fig. 3) and the lowest average values of MIC (Table 3) against all *E. coli* isolates as well.

To understand which type of compounds is determinant for these bioactivities, we performed a determination of the Pearson´s correlation (between the MICs, IC_{50} values and average content of each polyphenol detected in samples), and the results presented in Table 4 indicate that MICs and IC_{50} values are highly dependent on the combined effect of the different polyphenols. Nonetheless, some compounds, such as sakuranetin, isosakuranetin, neochlorogenic and chlorogenic acid, catechin and the epicatechin derivative, *p*-coumaric acid and the *p*-coumaroylquinic acid derivative, had shown to be highly preponderant for IC_{50} and MIC average levels (Table 4). These compounds presented the highest negative correlation coefficients, which means that the higher they were, the lower the IC50 and MIC values were, and thus the highest TAA and antimicrobial activity. Our findings are important since they reinforce some recent conclusions about the antioxidant capacity and antimicrobial effect of such compounds (Wong *et al.*, 2011; Shimizu *et al.*, 2012). The ability of these compounds to modify cell structures and cause deformations in their structure, and as a consequence the loss of functionality, was recently observed (Denev *et al.*, 2014). Their chemical structure is based on two, three or more aromatic hydroxyl compounds, as we have detected (see Fig. 4), and a system of delocalized electrons is very effective against several microorganisms due to its interference with cellular membranes (Ultee *et al.*, 2002). Compounds with two or more aromatic hydroxyl groups have high affinity to bacterial membranes, causing destabilization of the outer membrane, leading to a decrease in the membrane potential and thus, an additional decrease in the pH as a result of a hydroxyl group presence and a system of delocalized electrons (Ultee *et al.*, 2002). Tsai *et al.* (2012) also found a high affinity of the neochlorogenic acid to bacterial cellular membranes, interfering with both, the membrane and cytoplasmic organelles and acting as protoplasmic poisons, thus, being highly effective in supressing the Gram-negative bacterial isolates. Similar situation was observed by Park *et al.* (2014) for several other microorganisms and different Gram-negative isolates. Thus, we can conclude that the presence of sakuranetin, isosakuranetin, neochlorogenic and chlorogenic acid were highly determinant for the antibacterial activity exhibited by sweet-cherry extracts against *E. coli*. These results explain, scientifically, why in several countries like Portugal, the stems from sweet-cherry are largely used in folkloric and traditional medicine as tea, decoctions or infusions, for depurative, anti-inflammatory and antimicrobial therapy, particularly in the treatment of several urinary tract infections, mostly caused by *E. coli* strains. Based on our results, the pointed bioactivities for sweet-cherry stems are highly correlated with the reported flavonoids and phenolic acids. To conclude, the bioactivities found in the current study can be the starting point for further exploitation of such by-products as additives or supplements. Our results seem to demonstrate the usefulness of sweet-cherry wastes to extract bioactive compounds with enhanced bioactivity, particularly phenolic acids and flavanones.

Acknowledgements

The authors acknowledge the financial support provided by the FCT-Portuguese Foundation for Science and Technology (Alfredo Aires-SFRH/BPD/65029/2009) under the QREN-POPH-Tipologia 4.1-Formação Avançada program.

Conflict of Interest

No conflict of interest declared.

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