Sweet cherries from Fundão possess antidiabetic potential and protect human erythrocytes against oxidative damage

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1. Introduction

In the last few years, the increasing reports about a balanced diet, rich in fruits and vegetables, have shown this to be a good source of phytochemicals with great antioxidant capacity which is associated with improved health, playing also an important role in the prevention of chronic diseases, like cardiovascular diseases, Alzheimer’s disease, diabetes and cancer (Slavin & Lloyd, 2012). Sweet cherries (Prunus avium Linnaeus (L.)) are one of the fruits that have been largely studied, belonging to the same genus and family of peaches, apples, apricots and plums. In Portugal there is a long tradition of cultivating cherries, particularly in the northeast of the country, in county of Fundão, producing around 15,000 tons of cherries annually (Serra, Duarte, Bronze, & Duarte, 2011). They are mainly consumed as fresh, alternatively they can be processed and commercialized alone or incorporated in several products, such as dry (with or without sugars), frozen, jams, concentrate juices, powdered and canned (Ferretti, Bacchetti, Belleggia, & Neri, 2010).

Sweet cherries are characterized by a reduced level of calories, high contents of water (around 80%) and absence of sodium. They also present a considerable content of organic acids, fibers, vitamins, potassium, fatty acids, and phytochemical compounds, like volatiles, carotenoids, flavonoids (flavonols, flavan-3-ols, flavanones, flavones and, anthocyanins), hydroxycinnamic and hydroxybenzoic acids (Duarte & Silva, 2014). Among these compounds, special interest has been focused on polyphenols due their great capacity to capture reactive oxygen and nitrogen species, and also due to the strong anti-inflammatory activity shown (Serra et al., 2011). The presence of these compounds in sweet cherries is regulated by climatic conditions, genotype, fruit maturity and storage conditions (Gonçalves et al., 2004).

Several works reported that daily intake of sweet cherries alleviates gout and arthritis pain, neurological, gastrointestinal, tumoral and cardiovascular pathologies (Duarte & Silva, 2014; Ferretti et al., 2010; Jakobek, Seruga, Novak, & Medvidovic-Kosanovic, 2007), and prevents diabetes, since studies reported that some phenolic compounds can inhibit α-glucosidase activity, described as an enzyme responsible for the digestion of carbohydrates in absorbable monosaccharides (Duarte & Silva, 2014).
Silva, 2014; Ferretti et al., 2010; Silva & Teixeira, 2015; Teixeira & Silva, 2013).

These findings suggest that sweet cherries can minimize the accumulation of free radicals and could be used as effective drugs or as functional food. Therefore, the aim of this work was to determine the coloured and non-coloured phenolic profile of five sweet cherries from Fundão region (Portugal) by liquid chromatography with diode array detection (LC-DAD) and evaluate their biological potential. The antioxidant activity was performed against DPPH and nitric oxide radicals (‘NO), the α-glucosidase inhibitory potential was also evaluated, and the protection against induced oxidative damage in human erythrocytes by sweet cherries was evaluated through microassays. As far as we know, this is the first study about the capacity of sweet cherries to inhibit α-glucosidase enzyme, and to protect human erythrocytes against peroxyl radicals (ROO•), concerning inhibition of hemoglobin oxidation and erythrocyte hemolysis.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. Cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, pelargonidin-3-O-rutinoside and peonidin-3-O-rutinoside were from Extrasynthese (Genay, France). 3-O-Caffeoylquinic acid, p-hydroxybenzoic acid, p-coumaric acid, kaempferol-3-O-glucoside, quercetin, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, quercetin-3-O-galactoside, catechin, epicatechin and caffeic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), α-glucosidase from Saccharomyces cerevisiae (type I, lyophilized powder), phosphate-buffered saline (PBS), trypan blue and 2,2′-azobis (2-ethylpropionamide) dihydrochloride (AAPH), nitrotyetrazolium blue (NBT), α-glucosidase from Saccharomyces cerevisiae (type I, lyophilized powder), phosphate-buffered saline (PBS), trypan blue and 2,2′-azobis (2-ethylpropionamide) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-naphthyl)ethylene diamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside (pNPG) and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Methanol and acetonitrile were from Fisher Chemical (Leicestershire, United Kingdom). Water was deionized using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid).

2.2. Cherry samples

Five sweet cherry cultivars (1 kg each), namely Saco, Sweetheart, Satin, Maring and Hedelfinger were collected from Fundão region (Portugal) at the same stage of ripeness, by hand, between May and June of 2015. The fruits were immediately transported to the laboratory facilities, where pits were removed and separated from the pulp. The cherries’ pulp was immediately frozen with liquid nitrogen and maintained at −20 °C. Then, they were lyophilized and powdered (mean particle size lower than 910 μm), being divided into three aliquots, extracted and analysed separately.

2.3. Phenolic compounds

2.3.1. Extraction

The non-coloured and coloured phenolic compounds were extracted according to the procedure described by Silva and Queiroz (2016), with some modifications. Aliquots of 1 g of powder sample were weighed and extracted with 20 mL of EtOH (70%) along 2 h, under stirring after flushing with nitrogen to avoid oxidations. This extract was centrifuged at 4000 rpm during 10 min. Subsequently, the material was again extracted during 15 min with 100 mL of EtOH (70%). Both supernatants were evaporated to dryness under reduced pressure at 30 °C. The resulting extract was dissolved with 50 mL of deionized water and placed into the column. The C18 solid-phase extraction (SPE) column (70 mL/10,000 mg; Macherey-Nagel) was previously conditioned with 20 mL of ethyl acetate, 20 mL of ethanol and 20 mL of 0.01 mol/L HCl. After passing the sample (non-coloured and coloured phenolics), the column was washed with 3 mL of 0.01 mol/L HCl. Then, fraction I (non-coloured phenolics) was eluted with 20 mL of ethyl acetate and placed in an erlenmeyer. Fraction II (anthocyanins) was eluted with 40 mL of ethanol containing 0.1% HCl and placed in a second erlenmeyer. Both fractions were evaporated under reduced pressure, and the dried extracts obtained were re-dissolved with 4 mL of methanol (fraction I) and in 20 mL of acidified water, pH 3.0 (fraction II) using a membrane filter (0.45 μm). 20 μL of each sample were analysed on a LC model Agilent 1260 system (Agilent, Santa Clara, California, USA) using a Nucleosil® 100-5 C18 column (25.0 cm × 0.46 cm; 5 μm particle size waters; Macherey-Nagel, Düren, Germany). Detection was achieved with an Agilent 1260 Infinity Diode Array Detector (DAD) using the ChemStation software supplied by Agilent Technologies (Waldbronn, Germany).

2.3.2. Anthocyanins

The method used for anthocyanins (fraction II) extraction was based on Silva and Queiroz (2016). The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and water/formic acid/acetonitrile (40:10:50, v/v/v; eluent B) using a gradient program as follows: from 10% to 25% B (10 min), from 25% to 31% B (5 min), from 31% to 40% (5 min), from 40% to 50% B (10 min), from 50% to 100% B (10 min), from 100% to 10% B (5 min). Total run time was 50 min. Flow rate was 0.8 mL/min. The injection volume was 20 μL. The compounds in each sample were identified by comparing their retention times and UV-VIS spectra in the 200–600 nm range with the library of spectra previously compiled by the authors. Anthocyanin quantification was achieved by the absorbance recorded in the chromatograms relative to external standards at 500 nm. Compounds unknown 1 and unknown 2 were quantified as cyanidin-3-O-rutinoside.

2.3.3. Non-coloured phenolics

The method for quantification of the non-coloured phenolics (fraction I) was previously described by Silva and Queiroz (2016). The mobile phase used is composed by 2% (v/v) acetic acid in water (eluent A) and 0.5% (v/v) acetic acid in water and acetonitrile (50:50, v/v, eluent B). The solvent system starting with 10% of B, and installing a gradient to obtain: 24% B at 20 min, 30% B at 40 min, 55% B at 60 min, 70% B at 80% B at 70 min), 100% B at 75 min, and maintain 100% B isocratic during 5 min (80 min). The established solvent flow rate was 1.0 mL/min. The injection volume was 20 μL. Spectral data from all peaks were accumulated in the range of 200–400 nm. Phenolic compound quantification was achieved through the absorbance recorded in the chromatograms relative to external standards at 280 nm for flavan-3-ols and hydroxybenzoic acids, 320 nm for hydroxycinnamic acids and 350 nm for flavonols. The compounds in each extract were identified by comparing their retention times and UV-VIS spectra with those of authentic standards. The hydroxybenzoic acid derivative was quantified as p-hydroxybenzoic acid. The 3-O-cafeoylquinic acid and hydroxycinnamic acid derivative were quantified as 5-O-cafeoylquinic acid, p-Coumaric acid derivative 1, p-coumaroylquinic acid and p-coumaric acid derivative 2 were quantified as p-coumaric acid. Catechin derivative was quantified as catechin.

2.4. Biological assays

The extract used for the biological assays was obtained from the mixture of both fractions I and II used for the evaluation of the phenolic compounds described above. The fractions were dried, after which it was observed an average yield of 3.1 ± 0.006% from the starting dry material.
2.4.1 Evaluation of antioxidant activity

2.4.1.1 DPPH assay. The ability of sweet cherries extracts to act as free radicals scavengers against DPPH was determined following a described procedure (Silva et al., 2014). For each extract, seven different dilutions were prepared, placed into a 96-well plate, and read at 562 nm. Ascorbic acid was used as positive control. Three experiments were performed in triplicate.

2.4.1.2 Nitric oxide assay. The activity against NO was determined as described in previous works (Silva et al., 2014). The chromophore formed with Griess reagent was read at 526 nm. For each extract, seven different concentrations were prepared in a 96-well-plate. Ascorbic acid was used as positive control. Three experiments were performed in triplicate.

2.4.2 α-Glucosidase inhibitory activity

The inhibition of α-glucosidase activity was determined at 405 nm, based on Ellman’s method previously described (Silva & Teixeira, 2015). For each extract, six different concentrations were tested. Acarbose was used as positive control. Three experiments were performed in triplicate.

2.5 In vitro ROO•-induced oxidative damage in human erythrocytes

For the evaluation of the in vitro ROO•-induced oxidative damage in human erythrocytes only one cultivar was used. Saco cherry was selected taking into consideration that it is the most important cultivar in the Fundão region.

2.5.1 Isolation of human erythrocytes

Venous human blood was collected from randomized patients of Centro Hospitalar de Coimbra (Covilha), by antecubital venipuncture into K3EDTA vacuum tubes. Erythrocytes were isolated based on the procedure described by Chisté, Freitas, Mercadante, & Fernandes (2014). Briefly, the collected blood was transferred to sterile conic tubes (15 mL), mixed with 6 mL of PBS (pH 7.4) and centrifuged at 1500×g for 5 min at 4 °C. After centrifugation, the supernatant was discarded; the erythrocytes were washed with 6 mL of PBS and centrifuged again. This procedure was repeated twice and the resulting supernatant (300 μL) was placed in a 96-well plate and the absorbance was read at 560 nm (Mariutti, Rodrigues, Chisté, Fernandes, & Mercadante, 2014). Five experiments were performed in duplicate.

2.5.2 Inhibition of hemoglobin oxidation

The inhibition of hemoglobin (Hb) oxidation was evaluated by monitoring the effects of the lyophilized Saco extract on the formation of metHb (Chisté, Freitas, Mercadante, & Fernandes, 2014) after the reaction of oxyhemoglobin (HbO2) with ROO• generated by AAPH. The extract was dissolved in PBS (6.3–100 μg/mL, final concentration), mixed with the suspension of human erythrocytes (1250 × 106 cells/mL, final density) and incubated at 37 °C in a water-bath, for 30 min, under slow agitation (∼50 rpm). After incubation, AAPH (50 mM, final concentration) was added to the media and then incubated in the same conditions described above for 4 h. The entire volume of the reaction mixture was centrifuged at 1500×g for 5 min at 4 °C. The supernatant (300 μL) was placed in a 96-well plate and the absorbance was read at 540 nm (Mariutti, Rodrigues, Chisté, Fernandes, & Mercadante, 2014). Five experiments were performed in duplicate.

2.5.3 Inhibition of hemolysis

ROO• were generated by AAPH and the prevention of ROO•-induced hemolysis of human erythrocytes was evaluated by monitoring the release of Hb after membrane disruption caused by the hemolytic process, according to the optimized procedure described by Chisté et al. (2014). Briefly, six different concentrations of the lyophilized Saco extract dissolved in PBS (16–500 μg/mL, final concentration) and the suspension of human erythrocytes (1775 × 106 cells/mL) were incubated at 37 °C in water-bath during 30 min, under slow agitation (∼50 rpm), followed by the addition of AAPH solution (17 mM) and incubated again for 3 h in the same conditions described before. After incubation, the entire volume of the reaction mixture was transferred to 1.5 mL conic microtubes and centrifuged at 1500×g for 5 min at 4 °C. The supernatant (300 μL) was placed in a 96-well plate and the absorbance was obtained at 540 nm. Five experiments were performed in duplicate.

2.6 Statistical analysis

Statistical comparison was made using one-way ANOVA and the means were classified by Tukey’s test at a 95% level of significance. Differences were considered significant for P < 0.05. To determine the correlation between the antioxidant activity methods and the contribution of the total phenols, Pearson’s correlation coefficients were calculated. All analyses were performed using Graph Pad Prism Version 6.01.

3. Results and discussion

3.1 Phenolic composition

3.1.1 Anthocyanins

The analysis by LC-DAD allowed the identification of four compounds being quantified six anthocyanins: unknown 1 (1), cyanidin-3-O-glucoside (2), cyanidin-3-O-rutinoside (3), unknown 2 (4), pelargonidin-3-O-rutinoside (5) and peonidin-3-O-rutinoside (6) (Fig. 1 and Table 1).

All of these compounds were previously described in sweet cherry cultivars (Ballistreri et al., 2013; Jakobek et al., 2007; Serra et al., 2011), except for both unknown 1 and 2, even though we cannot identify the compounds, they are here reported for the first time (Table 1). Despite the differences observed in the amounts of each anthocyanin, the analysed samples exhibited similar profile. The anthocyanin identified as unknown 1 was only identified in Saco and Hedelfinger (Table 1).

Fig. 1. A profile of Saco sweet cherry obtained by LC-DAD. Detection at 500 nm. (1) unknown 1, (2) cyanidin-3-O-glucoside, (3) cyanidin-3-O-rutinoside, (4) unknown 2, (5) pelargonidin-3-O-rutinoside and (6) peonidin-3-O-rutinoside, according to Table 1.
Table 1

<table>
<thead>
<tr>
<th>Anthocyanins of sweet cherries from Fundão (μg/g of hylphillized - sample)</th>
<th>Regression equations</th>
<th>R²</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
<th>Interday precision (CV%)</th>
<th>Repeatability (CV%)</th>
<th>Recovery (%)</th>
<th>Saco (μg/g)</th>
<th>Maring (μg/g)</th>
<th>Hedelfinger (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown 1</td>
<td>Y = 37.77X + 23.53</td>
<td>0.9991</td>
<td>0.63</td>
<td>0.47</td>
<td>1396</td>
<td>1336</td>
<td>31.26</td>
<td>24.58</td>
<td>18.12</td>
<td>15.63</td>
</tr>
<tr>
<td>Cyanidin-3-O-glucoside</td>
<td>Y = 14.24X + 2.68</td>
<td>0.9993</td>
<td>0.67</td>
<td>0.47</td>
<td>1396</td>
<td>1336</td>
<td>31.26</td>
<td>24.58</td>
<td>18.12</td>
<td>15.63</td>
</tr>
<tr>
<td>Cyanidin-3-O-rutinoside</td>
<td>Y = 14.24X + 2.68</td>
<td>0.9993</td>
<td>0.67</td>
<td>0.47</td>
<td>1396</td>
<td>1336</td>
<td>31.26</td>
<td>24.58</td>
<td>18.12</td>
<td>15.63</td>
</tr>
<tr>
<td>Pelargonidin-3-O-rutinoside</td>
<td>Y = 39.22X + 20.56</td>
<td>0.9990</td>
<td>0.60</td>
<td>1.84</td>
<td>0.83</td>
<td>13.48</td>
<td>9.47</td>
<td>nq</td>
<td>16.02</td>
<td>15.71</td>
</tr>
<tr>
<td>Peonidin-3-O-rutinoside</td>
<td>Y = 58.90X + 32.24</td>
<td>0.9991</td>
<td>0.40</td>
<td>1.22</td>
<td>1.28</td>
<td>9.38</td>
<td>23.40</td>
<td>8.66</td>
<td>42.62</td>
<td>3.21</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation of three assays. Σ is the sum of the determined anthocyanins; nq, not quantified.

On the other hand, pelargonidin-3-O-rutinoside was identified in all samples, except in Satin (Table 1).

The linearity was obtained with standard solutions at seven different concentrations, selected as representative of the range of concentrations of the compounds in sweet cherries. The calibration plots showed a good correlation, as indicated by correlation coefficient (R²) with values higher than 0.99 for all anthocyanins (Table 1). The limit of detection (LOD = 3S₀ / b), and limit of quantification (LOQ = 10S₀ / b) (S₀ is the standard deviation of the signal-to-noise ratio of a low concentration standard and b is the slope of the calibration curve) (Oliveira et al., 2010) are also shown in Table 1. The LOD and LOQ measured in sweet cherry cultivars ranged between 0.21 and 0.63 ng/mL and 0.63–1.91 ng/mL, respectively. These results are in agreement with a previous work that presented LOD and LOQ for anthocyanins to be around 0.30 and 0.50 ng/mL and 0.10 and 1.25 ng/mL, respectively (Sandhu, Edirisinghe, Burton-Freeman, & Zweigenbaum, 2016).

To evaluate recovery, aliquots of cyanidin-3-O-rutinoside standard solution were treated with the same method and quantified by LC-DAD, obtaining a recovery of 101.93 ± 0.27%. Repeatability was performed by analysing the same samples five times in the same day by the same analyst. The coefficients of variation obtained were lower than 5% (Table 1), proving that the repeatability of the procedure was satisfactory (Bayram, Ozcelik, Schultheiss, Frank, & Rimbach, 2013). Furthermore, the interday precision was determined by analysing the samples on five different days (one injection per day), and coefficients of variation found were lower than 14% (Table 1), indicating that interday precision was within the established parameters.

The total amounts of anthocyanins ranging between 1076.97 and 2183.55 μg/g of lyophilized sweet cherries. Maring was the richest cultivar followed by Hedelfinger and Saco, being Satin the poorest one (Table 1). In a general way, our results are in accordance with Serra et al. (2011), where anthocyanins content varied between 560 and 3720 μg/g expressed as dry weight (dw) of fruit.

Cyanidin-3-O-rutinoside was the major compound identified in all sweet cherry cultivars, representing 87.7%–91.9% of total contents of anthocyanins, and 42.5%–68.6% of total phenolic compounds (Tables 1 and 2). Our data is in accordance with other previous works that reported cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside as the main anthocyanins present in sweet cherries (Kelebek & Selli, 2011; Serra et al., 2011). As far as we know, this is the first report about the anthocyanin profile of Satin cultivar.

Comparing the sweet cherries content with other red fruits, they were richer in anthocyanins than tart cherries (Prunus cerasus L.) (618.1 μg/g expressed as dw), and also presented higher contents in cyanidin-3-O-rutinoside, however tart cherries presented other anthocyanins not reported in sweet cherries, such as cyanidin-3-glucosylrutinoside and cyanidin-3-sophoroside (Seymour et al., 2008). The Portuguese red grapes namely Jaen, Alfrochadeiro and Syrah showed similar anthocyanin contents to sweet cherries, except Touriga, that showed two times more anthocyanins than the anthocyanin-rich Maring (Table 1). The main anthocyanin found in grapes was malvidin-3-O-glucoside (Silva & Queiroz, 2016). Sweet cherries also showed a similar content to blackberry fruits (Rubus fruticosus L.) (1760 μg/g expressed as dw). Cyanidin-3-O-glucoside was reported as the main one in these fruits, followed by cyanidin-3-xylolside, cyanidin-3-malonylglicoside, cyanidin-3-dioxalylglucoside and cyanidin-3-sambubioside (Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014).

3.1.2. Non-coloured phenolics

In respect to non-coloured phenolic compounds, the analysis of sweet cherries by LC-DAD allowed the identification and quantification of seventeen non-coloured different phenolics, comprising one hydroxybenzoic acid (1), eight hydroxycinnamic acids (peaks 2–11), three flavan-3-ols (peaks 5, 10 and 12) and five flavonols (peaks 13–17) (Fig. 2 and Table 2).
and quercetin were only identified in cherry samples. Nevertheless, the five cultivars showed qualitative and quantitative differences (Table 2). Quercetin-3-O-galactoside was only detected in Sweetheart and Hedelfinger. Additionally, p-coumaric acid and quercetin were only identified in Saco and Hedelfinger. Catechin was not detected in Satin and Hedelfinger.

Calibration curves were obtained with standard solutions at seven different concentrations, selected as representative for the range of compound concentrations in sweet cherries. The calibration plots showed a good correlation, as indicated by R² values higher than 0.99 for all non-coloured phenolics (Table 2). The LOD and LOQ determined ranged between 389.10 and 2024.44 ng/mL and 0.50–3.59 ng/mL, respectively (Table 2). These results are in accordance with previous works which reported the 3-O-caffeoylquinic acid as the main non-coloured phenolic compound, being 3-O-caffeoylquinic acid the major one found in all the studied cherry samples, except in Satin, where p-coumaroylquinic acid was the predominant hydroxycinnamate.

These results are in accordance with previous works which reported the 3-O-caffeoylquinic acid as the main non-coloured phenolic in Saco, Maring and Sweetheart cultivars (Hayaloglu & Demir, 2016; Serra et al., 2011).

Concerning flavonoids (flavan-3-ols and flavonols), epicatechin (flavan-3-ol) was the one presenting higher amounts in Saco, corresponding to 86.3% of total non-coloured phenolic compounds, being 3-O-caffeoylquinic acid the major one found in all the studied cherry samples, except in Satin, where p-coumaroylquinic acid was the predominant hydroxycinnamate.
Table 2
Non-coloured phenolic contents of five sweet cherries from Fundão (μg/g of lyophilized sample).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression equations</th>
<th>R²</th>
<th>LOD (ng/mL)</th>
<th>CV%</th>
<th>3.0 Saco</th>
<th>2.5 Saco</th>
<th>2.5 Sweetheart</th>
<th>2.5 Satin</th>
<th>2.5 Bing</th>
<th>2.5 Maring</th>
<th>2.5 Hedelfinger mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxybenzoic acid derivative</td>
<td>Y = 20.16X + 47.964</td>
<td>0.9953</td>
<td>1.18</td>
<td>3.59</td>
<td>0.964</td>
<td>3.32</td>
<td>0.169</td>
<td>0.05</td>
<td>0.77</td>
<td>0.05</td>
<td>0.161 ± 0.05</td>
</tr>
<tr>
<td>O-Coumaroylquinic acid</td>
<td>Y = 142.47X + 153.30</td>
<td>0.9997</td>
<td>0.16</td>
<td>0.50</td>
<td>1.26</td>
<td>10.36</td>
<td>175.03</td>
<td>2.69</td>
<td>3.46</td>
<td>1.24</td>
<td>349.12 ± 1.06</td>
</tr>
<tr>
<td>Catechin</td>
<td>Y = 21.72X + 16.74</td>
<td>0.9983</td>
<td>1.09</td>
<td>3.33</td>
<td>1.70</td>
<td>3.45</td>
<td>14.70</td>
<td>0.32</td>
<td>4.97</td>
<td>0.22</td>
<td>4.97 ± 0.22</td>
</tr>
<tr>
<td>O-Caffeoylquinic acid</td>
<td>Y = 57.75X + 74.07</td>
<td>0.9997</td>
<td>0.41</td>
<td>1.25</td>
<td>2.07</td>
<td>76.41</td>
<td>26.98</td>
<td>0.92</td>
<td>100.99</td>
<td>0.40</td>
<td>100.99 ± 0.40</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Y = 136.66X + 18.19</td>
<td>0.9999</td>
<td>0.17</td>
<td>0.52</td>
<td>5.40</td>
<td>11.19</td>
<td>2.67</td>
<td>0.21</td>
<td>17.73</td>
<td>0.12</td>
<td>17.73 ± 0.12</td>
</tr>
<tr>
<td>O-Coumaric acid derivative 2</td>
<td>Y = 142.47X + 153.30</td>
<td>0.9997</td>
<td>0.16</td>
<td>0.50</td>
<td>2.53</td>
<td>12.69</td>
<td>3.45</td>
<td>0.11</td>
<td>17.83</td>
<td>0.13</td>
<td>17.83 ± 0.13</td>
</tr>
<tr>
<td>Catechin derivative</td>
<td>Y = 21.72X + 16.74</td>
<td>0.9983</td>
<td>1.09</td>
<td>3.33</td>
<td>9.86</td>
<td>7.80</td>
<td>4.07</td>
<td>0.31</td>
<td>13.86</td>
<td>0.15</td>
<td>13.86 ± 0.15</td>
</tr>
<tr>
<td>O-Rutinoside</td>
<td>Y = 38.29X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>389.10 ± 0.05</td>
</tr>
<tr>
<td>Quercetin-3-glucoside</td>
<td>Y = 59.44X + 8.18</td>
<td>0.9998</td>
<td>0.40</td>
<td>1.21</td>
<td>1.15</td>
<td>7.06</td>
<td>2.67</td>
<td>0.22</td>
<td>13.50</td>
<td>0.15</td>
<td>13.50 ± 0.15</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation of three assays. *P > 0.05 is indicated as ns, compared to 2.5 Sweetheart.

The first study that reported the non-coloured phenolic profile of Satin cultivar.

Compared with other red fruits, sweet cherries showed less amounts of non-coloured phenolics than tart cherries (5103–7813 μg/g expressed as dw, respectively) (Kirakosyan, Seymour, Llanes, Kaufman, & Bolling, 2009). Furthermore, tart cherries present isorhamnetin-3-O-rutinoside (not identified in sweet cherries), querectin and kaempferol as the main compounds (Seymour et al., 2008). Red grapes proved to be poorer than sweet cherries (ranged between 343.80 and 1328.30 μg/g expressed as dw), being more abundant in epigallocatechin, catechin and quercetin-3-O-glucoside (Silva & Queiroz, 2016). As sweet cherries, blueberries (total amount of non-coloured phenolics = 26.700 μg/g expressed as dw) are very rich in hydroxycinnamic acids, mainly caffeic acid, but presenting lower amounts of quercetin than sweet cherries (Jakobek, Seruga, Seruga, Novak, & Medvidović-Kosanović, 2009).

3.2. Antioxidant potential

Natural antioxidants present in fruits and vegetables have gained increasing interest among the scientific community and consumers, because epidemiological studies have indicated that regular consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006; Miguel, 2011).

DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule, considered an easy standard colorimetric method for the evaluation of antioxidant properties of pure compounds and extracts, giving us a general screening of antioxidant potential (Ebrihamzadeh, Nabavi, Nabavi, Eslami, & Rahmani, 2010; Teixeira & Silva, 2013). All extracts exhibited a dose-dependent effect against DPPH. Hedelfinger, Satin and Saco were the most active (IC₅₀ = 12.1 ± 0.37; 141.0 ± 0.43 and 162.6 ± 0.69 g/mL of dried extract, respectively) (Fig. 3A and Table 3), displaying similar activity to the positive control, ascorbic acid (IC₅₀ = 16.92 ± 0.69 g/mL).

Our values are in accordance with Prvulović, Popović, Malenič, Ljubojević, and Ognjanov (2011), who reported that the aqueous:acetone (70:30) extract of Hedelfinger showed great capacity to scavenge DPPH' (showing an activity of 86.94% at 0.02 g/mL). Comparatively with other red fruits, sweet cherries revealed a higher activity than blackberry Ćaranska Bestrna fruits (IC₅₀ ranged between 96.0 and 118.1 g/mL expressed as dried aqueous extract) (Ivanovic et al., 2014), and less activity than calafate fruits, blueberries (IC₅₀ = 2.33 ± 0.21 μg/mL and 3.32 ± 0.18 μg/mL expressed as dried aqueous extract, respectively) and strawberries (Fragaria × ananassa Duch.) (IC₅₀ = 7.6 ± 2.1 μg/mL expressed as dried aqueous extract) (Brito, Areche, Sepúlveda, Kennelly, & Simirgiotis, 2014; Mandave, Rani, Kuvalake, & Ranjekar, 2013). Furthermore, the activities shown by Hedelfinger, Satin and Saco were similar to bilberries (Vaccinium myrtillus L.) (IC₅₀ = 14.87 ± 0.52 μg/mL expressed as dried methanolic extract) (Güder, Gür, & Engin, 2015).

The extracts were then tested against NO, a free radical present in our organism and if overproduced has a negative impact in mitochondria and proteins, reacting with oxygen and superoxide reactive species, producing more toxic radicals like peroxynitrite and ROO⁻, increasing cell damage. Additionally, it can activate pro-inflammatory transcription factors, causing neurodegenerative and chronic diseases, as rheumatoid arthritis, diabetes, atherosclerosis and cancer (Miguel, 2011; Silva et al., 2014). Along this assay all extracts exhibited a dose-dependent effect, being Maring, Saco and Hedelfinger (IC₅₀ = 140.91 ± 1.85, 176.68 ± 3.35 and 185.11 ± 1.52 μg/mL of dried extract, respectively) the most active (Fig. 3B and Table 3), displaying similar effect when compared to positive control, ascorbic acid (IC₅₀ = 162.66 ± 1.31 μg/mL). The positive effect of sweet cherry against NO had already been proved in a study performed with eighteen healthy men and women, consuming 280 g daily of Bing sweet cherries during 28 days. The results revealed a significant reduction (about 18%) in nitric oxide levels in the blood (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006).
Our samples are less active than Indian sweet cherries (IC₅₀ = 21.1 ± 2.31 µg/mL expressed as dried ethanolic extract) (Bhattacharjee, Kamal, & Roy, 2016), but more active than blueberries (IC₅₀ = 1500 µg/mL expressed as dried ethanolic extract) (Samad, Debnath, Ye, Hasnat, & Lim, 2014).

The antioxidant capacity of plant extracts is closely linked to their phenolic composition, whose anti-radical properties are known (Jakobek et al., 2007). In a general way, the antioxidant capacity of the phenolic composition, whose anti-radical properties are known (Tadera, Minami, Takamatsu, & Matsuoka, 2006). Anthocyanins, particularly cyanidin (and its glycosides), are great antioxidant molecules since they have more OH groups than the other compounds (Mendes, De Freitas, Baptista, & Carvalho, 2011). As mentioned above, all of the antioxidant assays were performed with hydroethanolic extracts of each sample. Additionally, phenolics were the only compounds identified in those extracts. As so, in order to search for possible correlations between the determined compounds and the antioxidant capacity displayed by sweet cherries’ extracts, Pearson’s test was performed considering the IC₅₀ values found in the both assays and the phenolic compounds content in each hydroethanolic extract. The results obtained indicate that the activity of all sweet cherries’ extracts against DPPH’ and ’NO was negatively correlated with the phenolic amounts. Nevertheless, individually, a little positive correlation was found between DPPH’ test, kaempferol-3-O-rutinoside (r = 0.4760) and peonidin-3-O-rutinoside (r = 0.4325). Additionally, p-coumaric acid derivative 1 (r = 0.9444), p-coumaroylquinic (r = 0.8646), and p-coumaric (r = 0.8012) and 5-O-cafeoylquinic (r = 0.6441) acids, and quercetin-3-O-glucoside (r = 0.8640) showed positive correlation with ’NO scavenging test. The possible presence of other non-determined active compounds like organic acids, volatiles, among others cannot be ignored, as they may contribute to increase the antioxidant potential.

### Table 3

IC₅₀ (µg/mL) values found in the antioxidant activity and α-glucosidase assays for sweet cherries dried extracts.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Saco</th>
<th>Sweetheart</th>
<th>Satin</th>
<th>Maring</th>
<th>Hedelfinger</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH’</td>
<td>16.24 ± 0.46</td>
<td>43.03 ± 0.53</td>
<td>14.10 ± 0.42</td>
<td>20.66 ± 0.52</td>
<td>12.12 ± 0.37</td>
</tr>
<tr>
<td>’NO</td>
<td>176.60 ± 3.35</td>
<td>227.90 ± 1.55</td>
<td>439.40 ± 2.44</td>
<td>40.91 ± 1.85</td>
<td>185.11 ± 1.52</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>10.79 ± 0.40</td>
<td>14.34 ± 0.56</td>
<td>16.31 ± 0.71</td>
<td>11.38 ± 0.48</td>
<td>10.25 ± 0.49</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation of three assay.

- Superscript letters indicate significant difference (P < 0.05).
- *P < 0.05 is indicated as vs Saco.
- *P < 0.05 is indicated as vs Satin.
- *P < 0.05 is indicated as vs Maring.
- *P < 0.05 is indicated as vs Hedelfinger.
Comparatively with other red fruits, the inhibition of α-glucosidase by sweet cherries superior to the activity reported for raspberries (IC\textsubscript{50} = 67.7 μg/mL expressed as dried ethyl acetate extract) (Yin et al., 2012), strawberries (IC\textsubscript{50} = 76.83 ± 0.93 μg/mL expressed as dried aqueous extract) (Mandave et al., 2013) and bilberries (Vaccinium myrtillus L) (IC\textsubscript{50} = 138.41 ± 1.05 μg/mL expressed as dried methanolic extract) (Güder et al., 2015).

The positive results obtained for sweet cherries against α-glucosidase may be attributed, at least partially, to their phenolic composition. Besides increasing antioxidant capacity, anthocyanins and non-coloured phenolics can inhibit α-glucosidase activity in both competitive and non-competitive ways, enhancing antidiabetic properties (Tadera et al., 2006). In this work, a direct relation between the phenolic content and antidiabetic capacity was also observed. For example, Hedelfinger, in addition to having the highest phenolic content, also revealed the highest inhibitory activity of α-glucosidase, followed by Saco (Tables 1 and 2).

3.4. Protective effects of Saco extracts against ROO’ in human blood samples

Erythrocytes are prime targets for free radical species owing to the presence of both high membrane concentration of polyunsaturated fatty acids and the oxygen transport closely linked with active hemoglobin molecules, which are promoters of reactive oxygen species (ROS) (Umbreit, 2007). Given so, in this experimental work and knowing the several benefits of detected cherry bioactive compounds, we evaluated for the first time the preventive effect of Saco sweet cherry extracts against ROO’-mediated toxicity generated by AAPH.

The oxidation of hemoglobin (resulting in methemoglobin (MHb), where the iron in the heme group is in the Fe\textsuperscript{3+} state and not as in normal state (Fe\textsuperscript{2+})) is not yet completely understood, but it is related to oxidative stress, in perturbations of protein interactions and damage in lipids, that makes the membrane of erythrocytes more susceptible to be degraded (Umbreit, 2007). MHb causes hypoxia events due to the inability of hemoglobin to bind or carry the oxygen, and causes an increase of ROS and reactive nitrogen species. MHb is also related to the lysis of erythrocytes and inflammatory processes, enhancing the release of interleukin (IL)-6 and IL-8, and E-selectin (adhesion molecule) at a cellular level (Umbreit, 2007).

Fig. 4A shows the protective effects of Saco extracts against hemoglobin oxidation in a concentration dependent manner (IC\textsubscript{50} = 38.57 ± 0.96 μg/mL). Saco extract showed values twelve times less effective than quercetin control (IC\textsubscript{50} = 3.10 μg/mL) analysed in the same conditions, reported as the most efficient phenolic compound against hemoglobin oxidation (Chisté et al., 2014).

Few studies were found about the capacity of fruit extracts to protect hemoglobin against oxidation. Relatively to other fruits, Saco extracts showed to be seven times more efficient to avoid hemoglobin oxidation than hydrophilic extracts of murici fruits (Byrsonima crassifolia), a fruit native to the North and Northeast regions of Brazil very rich in carotenoids (lutein and zeaxanthin), quercetin and gallic acid (IC\textsubscript{50} = 271 ± 44 μg/mL expressed as dried extract) (Mariuti et al., 2014).

The ability of flavonoids to prevent damage in erythrocytes is due to the OH substitutions: the more OH groups, the greater the capacity of scavenging these reactive species. To highlight the cyanidin derivatives (with and without a sugar molecule) role, which in addition to possessing many OH groups, they can easily bind to the membrane of erythrocytes, improving its strength, and enhancing its protection against oxidation (Bonarska-Kujawa, Sylwia, Żylka, Oszmałski, & Kleszczyńska, 2014). Relatively to non-coloured phenolics, Kitagawa, Sakamoto, and Tano (2004) reported that numerous flavonoids present in Saco sweet cherry, particularly quercetin, quercetin glycosides and (−)-epicatechin, can inhibit the hemoglobin oxidation. These flavonoids are able to oxidize the heme iron of erythrocytes, thereby inhibiting their enzymatic reactions and preventing oxidation.

Fig. 4. Inhibition of hemoglobin oxidation (A) and hemolysis (B) by Saco sweet cherry extract.

ROO’ generated by AAPH attacks the membrane of the erythrocytes from outside, leading to hemolysis. Saco extracts avoid hemolysis in a concentration dependent manner (IC\textsubscript{50} = 73.03 ± 1.48 μg/mL) (Fig. 4B), proving efficiency to scavenge radical species in the medium before they can attack the erythrocytes, protecting them from lysis. Despite their protective potential, Saco revealed to be 104 times less effective than quercetin control (the most efficient phenolic for erythrocytes protection reported until now) analysed in the same conditions (IC\textsubscript{50} = 0.7 μg/mL) (Chisté et al., 2014). Comparatively with other fruits, Saco sweet cherry proved to be five times more efficient than strawberry fruits (Arbutus unedo L) (IC\textsubscript{50} = 430.00 μg/mL expressed as dried aqueous extract) (Mendes et al., 2011), but six times less effective than Mexican grapes (Ruby Cabernet) (IC\textsubscript{50} = 11.62 μg/mL expressed as dried methanolic extracts) (García-Becerril et al., 2016).

It is well known that flavonoids enhance erythrocytes’ resistance against free radical species, mainly due to their capacity to capture them, by donating electrons. In addition, flavonoids also exhibit metal-chelation properties, quenching radicals formed in the aqueous phase before they can damage the erythrocytes’ membrane (Carvalho et al., 2010; Ebrahimzadeh et al., 2010), so it was expected that Saco was effective in the protection of erythrocytes against ROO’ generated by AAPH. These results are supported by another experimental work performed by Blasa, Candiracci, Accorsi, Placentini, and Piatti (2007) which proved that phenolics protect the erythrocyte membrane due to its liposolubility. This allows them to be strongly incorporated into the membrane and act as antioxidant agents, leading to minimization of the concentration of reactive species.
4. Conclusion

Considering the current interest in red fruits containing antioxidants and health-promoting phytochemicals as natural potential therapeutic agents, this work provides important information about the phenolic composition and biological potential of sweet cherries from Fundão (Portugal). A total of six anthocyanins and seventeen non-coloured phe-noleic compounds were quantified in sweet cherries, proving these to be a good source of bioactive compounds. Relatively to antioxidant activity, all samples showed great potential. Hedelipherger exhibited the best scavenging activity against DPPH, and Maring proved to be more effective against 'NO. Additionally, sweet cherries revealed great α-glucosidase inhibitory activity, being more effective than the control acarbose. Furthermore, Saco proved to be able to inhibit hemoglobin oxidation and erythrocyte hemolysis in a concentration dependent manner, obtaining promising values during the assays. The phenolics found in sweet cherries contribute to the observed effects, playing an important role in the prevention of oxidation and cellular damage. As so, the consumption of these fruits can have great importance for attenuate/mitigate the development of human diseases associated with oxidative stress. Nevertheless, more studies are needed to unravel other positive benefits of sweet cherries in human health.

Conflict of interest statement

The authors have declared no conflict of interest.

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