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Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit

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ABSTRACT

The present study reports the antioxidant properties of *Arbutus unedo* L. leaf and fruit extracts using different *in vitro* assays including (i) reducing power, (ii) scavenging effect on DPPH free radicals, and (iii) inhibitory effect on AAPH-induced hemolysis and lipid peroxidation in human erythrocytes. All assays demonstrated antioxidant efficiency for *A. unedo* L. aqueous extracts, being consistently higher in the leaf. EC_{50} values for reducing power and DPPH radical scavenging activities were, respectively, 0.318 ± 0.007 and 0.087 ± 0.007 mg/mL for leaf, and 2.894 ± 0.049 and 0.790 ± 0.016 mg/mL for fruit extracts. Under the oxidative action of AAPH, *A. unedo* leaf and fruit extracts protected the erythrocyte membrane from hemolysis (IC_{50} of 0.062 ± 0.002 and 0.430 ± 0.091 mg/mL, respectively) and decreased the levels of malondialdehyde, a breakdown product of lipid peroxidation (IC_{50} of 0.075 ± 0.014 and 0.732 ± 0.452 mg/mL, respectively). In accordance with antioxidant activity, phenolic content was found to be significantly higher in leaf extract. To our knowledge, this is the first time that the antioxidant activity of *A. unedo* species is evaluated using human biological membranes. Overall, our results suggest that *A. unedo* leaves are a promising source of natural antioxidants with potential application in diseases mediated by free radicals.

1. Introduction

Arbutus unedo L. (Ericaceae family), commonly known as strawberry tree, is an evergreen shrub or small tree endemic to Mediterranean region, but also encountered in other regions with hot summers and mild rainy winters (Celikel et al., 2008). Its fruits (berries) are spherical, about 2–3 cm in diameter, dark red, and tasty only when fully ripe in the autumn. *A. unedo* berries are rarely eaten as fresh fruits, but have considerable importance in local agricultural communities where they are used for the production of alcoholic beverages, jams, jellies, and marmalades (Alarcão-E-Silva et al., 2001; Pallauf et al., 2008). In traditional medicine, the fruits are recognized to have antiseptic, diuretic, and laxative effects, while the leaves are used as astringent, diuretic, urinary antiseptic, antidiarrheal, depurative and, more recently, in the therapy of hypertension, diabetes, and inflammatory diseases (Afkir et al., 2008; Mariotto et al., 2008; Bnouham et al., 2010).

Phytochemical studies showed that leaf extracts contain several phenolic compounds, like tannins, flavonoids, phenolic glycosides, among others (Males et al., 2006; Fiorentino et al., 2007; Pavlović et al., 2009; Tavares et al., 2010), as well as α-tocopherol (Kivçak and Mert, 2001). *A. unedo* berries are already known as a very good dietary source of antioxidants, including phenolic compounds (e.g. anthocyanins and other flavonoids, gallic acid derivatives and tannins), vitamins C and E, and carotenoids (Ayaz et al., 2000; Alarcão-E-Silva et al., 2001; Fortalezas et al., 2010; Males et al., 2006; Pawlowska et al., 2006; Pallauf et al., 2008; Tavares et al., 2010). Based on their chemical composition, it is reasonable to expect a high antioxidant activity for *A. unedo* fruit and leaf extracts. Therefore, in the present study, their antioxidant potential was assessed by the reducing power and DPPH radical scavenging activity assays. In addition, the biological significance of *A. unedo* antiradical activities was investigated using human cell-based model assays. The human erythrocyte was used as an *in vitro* model to study the oxidant/antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to free radical-mediated peroxidation, and is considered to be representative of the plasma membrane in general (Shiva Shankar Reddy et al., 2007). Erythrocyte lipid peroxidation may be involved in normal cell aging and it has been associated with a variety of

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pathological events (Ko et al., 1997; Sivilotti, 2004). In this study, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as the free radical initiator to induce oxidative damage in erythrocytes. Thermal decomposition at physiological temperature of AAPH generates peroxyl radicals in the aqueous phase (Niki, 1990), which can attack the erythrocyte membrane to induce lipid peroxidation. Since peroxidation of membrane lipids is a free radical chain reaction, the erythrocyte membrane is quickly damaged, leading to hemolysis. The protective effects of *A. unedo* leaf and fruit extracts were evaluated by inhibition of erythrocytes hemolysis and lipid peroxidation mediated by peroxyl radicals. To our best knowledge, this is the first time that the antioxidant activity of *A. unedo* species is evaluated using human biological membranes.

2. Materials and methods

2.1. Reagents

2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), gallic acid, L-ascorbic acid, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), thiobarbituric acid (TBA), and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO). Folin–Ciocalteu's phenol reagent was obtained from Fluka. Phosphate buffer solution (PBS) was obtained from Lonza Laboratories (Verviers, Belgium). All other chemicals were of analytical grade and obtained from Sigma (St. Louis, MO).

2.2. Samples

The leaves and fruits of *A. unedo* were collected in November of 2008, in the Natural Park of Montesinho (Bragança, Northeast of Portugal). Plant materials were collected in adult wild plants that grown freely without any treatment. Mature fresh and healthy leaves and fruits at the ripe stage (orange-red to strong red color) of *A. unedo* were randomly collected from a wild population. The samples were immediately frozen and freeze-dried (Ly-8-FM-ULE, Snijders) prior to extraction.

2.3. Extraction procedure

Dry powdered plant materials (5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min. The resulting extracts were then lyophilized and kept in a dessicator (in the dark), until analysis. The extraction yields were 39.3 ± 7.3% and 57.4 ± 1.2% for leaf and fruit, respectively.

2.4. Total phenolic measurement

The total phenolic content of leaf and fruit extracts was determined by using the Folin–Ciocalteu's phenol reagent, according to a previously described procedure (Costa et al., 2009). Briefly, 0.1 mL of water extract solution was mixed with 1 mL of Folin–Ciocalteu's phenol reagent and 5 mL of 20% sodium carbonate solution and the mixture adjusted to 10 mL with water. The reaction mixture was kept in dark at ambient temperature for 20 min, after which the absorbance was read at 735 nm. The total phenolic contents were determined from a standard curve of gallic acid and expressed as mg of gallic acid equivalents (GAE)/g of extract. All measurements were done in triplicate.

2.5. LC–DAD/ESI-MS analysis

A Finnigan Surveyor series liquid chromatograph, equipped with a Thermo Finnigan (Hypersil Gold) reversed-phase column (150 × 4.6 mm, 5 μm, C18) thermostated at 25 °C, was used. The samples were analysed using two solvents for elution: A – water/acetic acid (99:1; v/v) and B – water/acetonitrile/acetic acid (79:20:1; v/v/v). The gradient profile was: 0–55 min, 80–20% A, 55–70 min, 20–10% A, 70–90 min, 10–0% A, at a flow rate of 0.3 mL/min. The sample injection volume was 20 μL. The chromatographic column was washed with 100% B for 10 min and then stabilized with the initial conditions for another 10 min. Double-online detection was done by a photodiode spectrophotometer and mass spectrometry. The mass detector was a Finnigan LCQ DECA XP MAX (Finnigan Corp., San Jose, CA) quadrupole ion trap equipped with atmospheric pressure ionization (API) source, using electrospray ionization (ESI) interface. The vaporizer and the capillary voltages were 5 kV and 4 V, respectively. The capillary temperature was set at 325 °C. Nitrogen was used as both sheath and auxiliary gas at flow rates of 80 and 30, respectively (in arbitrary units). Spectra were recorded in negative ion mode between *m/z* 120 and 2000. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and an MS–MS of the most intense ion using relative collision energies of 30 and 60 V.

2.6. Reducing power assay

The reducing power of leaf and fruit extracts was determined according to the method of Oyaizu (1986). Various concentrations of sample extracts (1 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. At the end of incubation time, 2.5 mL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min in a refrigerated centrifuge (Centorion K24OR-2003). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations (SD). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against extract concentration.

2.7. DPPH radical scavenging activity assay

The capacity to scavenge the DPPH free radical was monitored according to a method reported by Hatano et al. (1988). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and allowed to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. The radical scavenging activity was calculated as percentage of DPPH discoloration using the equation: $\%\text{scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The assays were carried out in triplicate and the results expressed as mean ± SD. The extract concentration providing 50% inhibition (EC_{50}) was determined graphically by plotting the percentage of DPPH scavenging as a function of extract concentration.

2.8. Antioxidant assays using the human erythrocyte model

2.8.1. Preparation of human erythrocyte suspensions

Blood (5–10 mL) was obtained from healthy non-smoker adult individuals after informed consent. Human erythrocytes from citrated blood were immediately isolated by centrifugation at 1500 rpm for 10 min at 4 °C. After removal of plasma and buffy coat, the erythrocytes were washed three times with PBS (pH 7.4), and centrifuged at 1500 rpm for 10 min at 4 °C. After the final washing, the erythrocytes were resuspended using the same buffer to the desired hematocrit level.

2.8.2. Oxidative hemolysis inhibition assay

In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (dissolved in PBS; final concentration 50 mM). To study the protective effects of *A. unedo* aqueous extracts against AAPH-induced hemolysis, an erythrocyte suspension at 2% hematocrit was preincubated with the extracts of leaf (final concentrations of 50, 75, and 100 μg extract/mL diluted in PBS) and fruit (final concentrations of 400, 800 and 1600 μg extract/mL diluted in PBS) at 37 °C for 30 min, followed by incubation with and without 50 mM AAPH. This reaction mixture was shaken gently while being incubated at 37 °C for 4 h. In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with each extract) were used.

The extent of hemolysis was determined spectrophotometrically according to a method reported before (Ko et al., 1997; Costa et al., 2009; Magalhães et al., 2009; Carvalho et al., 2010). Briefly, aliquots of the reaction mixture were taken out at each hour of the 4 h of incubation, diluted with saline, and centrifuged at 4000 rpm for 10 min to separate the erythrocytes. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The hemolysis percentage was calculated using the formula: A/B × 100. The inhibitory concentration 50% (IC_{50}) at time 3 h was calculated by plotting the percentage of hemolysis inhibition *versus* the extract concentration. Ascorbic acid was used as a reference antioxidant compound. Five independent experiments were used for these calculations.

2.8.3. Lipid peroxidation inhibition efficiency

To study the inhibitory effect of *A. unedo* extracts on AAPH-induced lipid peroxidation in human erythrocytes, we maintained the experimental conditions described for the hemolysis inhibition assay with the exception that erythrocyte suspensions at 5.2% hematocrit were used. The extension of lipid peroxidation in erythrocytes was estimated by HPLC-UV quantification of malondialdehyde (MDA), a well-known carbonyl product of lipid peroxidation (Soares et al., 2004). Briefly, 250 μL of erythrocyte suspensions were taken at time 3 h and added to 25 μL of 0.2% BHT (in order to prevent further lipid peroxidation) and 1 mL of 1% TCA. The samples were then vortexed, centrifuged (10,000 rpm for 10 min, at 4 °C), and 500 μL of 1% TBA were added to equal volume of supernatant layer and allowed to stand at 95 °C during 45 min. After cooling at 4 °C, the samples were vortexed, centrifuged, and 50 μL of supernatants were injected on a HPLC system