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Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals

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ABSTRACT

Despite strawberry tree (*Arbutus unedo* L.) leaves had a long use in traditional medicine due to its antiseptic, diuretic, astringent and depurative properties, the potential of their antioxidant activity are still lacking. Our study goals to assess the antioxidant and free radical scavenging potential of water, ethanol, methanol and diethyl ether extracts of *A. unedo* leaves. Total phenols content was achieved spectrophotometrically using Folin–Ciocalteau reagent with gallic acid as standard. Antioxidant activity was evaluated using three different methods: reducing power of iron (III)/ferricyanide complex assay, scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and scavenging effect on superoxide radicals by using the PMS–NADH–nitroblue tetrazolium system. Ethanol extracts of *A. unedo* leaves were the highest in reducing power (IC_{50} 232.7 μg/mL) and DPPH scavenging effect (IC_{50} 63.2 μg/mL) followed by water extracts (with IC_{50} of 287.7 and 73.7 μg/mL, respectively); whereas diethyl ether extracts were the lowest. In the scavenging on superoxide radical assay, methanol extracts obtained the best results (IC_{50} 6.9 μg/mL). For all the methods tested the antioxidant activity was concentration dependent. In accordance with antioxidant activity, highest total phenols content were found in ethanol, followed by water, methanol and diethyl ether extract. The results indicated that *A. unedo* leaves are a potential source of natural antioxidants.

1. Introduction

The importance of oxygen-derived free radicals, commonly named reactive oxygen species (ROS), in health and disease is now recognized by every branch of medicine and biological science. ROS are chemically reactive molecules that are derived from the successive reduction of molecular oxygen to H_2O. They included free radicals, such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), and non-free-radical species, such as H_2O_2. Singlet oxygen ($^{-}O_2$) species are also forms of activated oxygen, among others (Aruoma, 1996a,b; Gülcin, 2007). The excessively produced ROS can injure cellular biomolecules such as proteins, carbohydrates, nucleic acids and lipids causing cellular and tissue damage (Aruoma, 1996a,b; Pulido et al., 2000). Overwhelming evidence indicates that ROS play a role in most major health problems of the industrialized world, including cardiovascular diseases, cancer, diabetes, neurological diseases, and atherosclerosis and are believed to be a major factor in aging (Finkel and Holbrook, 2000).

In recent years, the increasing interest around 'natural' products has encouraged the scientific community to obtain information about natural plant antioxidants and its importance in medicine, human nutrition and food industry (Liu and Ng, 2000; Wang, 2006). It has been demonstrated that plants contain many natural antioxidants compounds such as carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (Larson, 1988); which have been identified as a free radical or active oxygen scavengers (Zheng and Wang, 2001). Therefore, an appropriate dose of antioxidants derived from plants in the human diet can help to avoid the risk of contracting diseases where ROS are involved in its pathogenesis. Taking the traditional application form of *Arbutus unedo* L. into consideration, phytochemicals contents and in particular their antioxidant activity, are of considerable interested to investigated from the point of view of its use as a potential therapeutic agent against a wide range of human disease. Also, provide new scientific information for the further development of modern herbal medicines.

A. unedo, the strawberry tree, belongs to the Ericaceae family, and it is native of the Mediterranean climate (Celikel et al., 2008). In Portugal, the strawberry tree is mainly implanted in the south, being however present throughout all of the country in a dispersed way (Pedro, 1994). This species have been traditionally used as food, by using the arbutus berries in the production of alcoholic beverages, jams, jellies and marmalades (Alarcão-e-Silva et al., 2001; Pallauf et al., 2008); and as phytopharmaceuticals. For example, the fruits are well known in folk medicine as antiseptic, diuretic, and laxative, while the leaves are used as astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and more recently in the therapy of hypertension, diabetes and in the treatment of inflammatory diseases (Ziyyat et al., 1997; Ziyyat and

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Boussairi, 1998; Mariotto et al., 2008; Afkir et al., 2008). Indeed, phytochemical studies showed that leaf extracts contains several phenolic compounds, like tannins, flavonoids, phenolic glycosides, among others (Males et al., 2006; Fiorentino et al., 2007), as well as α-tocopherol (Kivçak and Mert, 2001). The composition of the berries is relatively well-known when compared to leaves. They contain several antioxidant molecules namely phenolic compounds (e.g. anthocyanins, gallic acid derivatives, tannins and flavonoids), vitamin C, vitamin E and carotenoids (Ayaz et al., 2000; Alarcão-e-Silva et al., 2001; Pawlowska et al., 2006; Males et al., 2006; Pallauf et al., 2008).

Based on their antioxidant composition, and while a detailed study is still lacking, we could reasonably anticipate a high-antioxidant activity of both berries and leaves of *A. unedo* (Wang and Lin, 2000). In a preliminary study, Pabuçcuoğlu et al. (2003) verified the antioxidant activity of *A. unedo* leaves ethanol and methanol extracts by $ABTS^{\cdot+}$[2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] method.

Therefore, the aim of this work is to allow a better knowledge of the antioxidant capacity of leaves from *A. unedo*. For this purpose, total phenols content, reducing power and radical scavenging activity [DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and $O_2^{\cdot-}$ scavenging] of leaves extracts were evaluated. The effect of the solvent polarity, used in the extracting procedure, on the yields and antioxidant activity has also discussed. The solvent systems tested included water, absolute methanol, ethanol and diethyl ether. It is expected from this study to assess the potentially of *A. unedo* leaves as a source of natural antioxidant for pharmaceutical and food application.

2. Materials and methods

2.1. Samples

The leaves of *A. unedo* L. were collected in January of 2009, in the Natural Park of Montesinho (Bragança, northeastern region of Portugal). The samples were immediately frozen and freezed dried (Ly-8-FM-ULE, Snijders) prior to extraction.

2.2. Extract preparation

Three powdered sub samples (~5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman no. 4 paper. The extracts (water extracts – WE) were then evaporated under vacuum (rotary evaporator Büchi R-210), and dissolved in phosphate buffer (KH_2PO_4, pH 7.4) to a final concentration of 50 mg/mL.

For ethanolic (EE), methanolic (ME) and diethyl ether extraction (DEE), 1.5 g of lyophilized leaves was extracted three times with 25 mL of the tested solvents and filtered through Whatman no. 4 paper. The extracts were then evaporated under vacuum (rotary evaporator Büchi R-210), dissolved in phosphate buffer (KH_2PO_4, pH 7.4) to a final concentration of 50 mg/mL and stored in the dark at 4 °C for further use.

2.3. Antioxidant activity

2.3.1. Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma (St. Louis, MO, USA). Folin–Ciocalteau's phenol reagent was obtained from Fluka. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol and n-Hexane were obtained from Panreac (Spain). Diethyl ether and 96% ethanol were purchased from Riedel-de Haën. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.3.2. Total phenols

Total phenols quantifications were achieved according to Singleton and Rossi (1965), with minor modification. Thus, 1 mL of the extract solution was mixed with 1 mL of Folin–Ciocalteau's phenol reagent. The mixture was shaken vigorously and left to stand for 3 min. After that, 1 mL of a saturated solution of sodium carbonate was added and the total volume was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after what the absorbance was read at 725 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. Galic acid was used as standard, being the results expressed in mg of gallic acid equivalents (GAE)/g of extract.

2.3.3. Reducing power assay

The reducing power was determined according to a described procedure (Berker et al., 2007). 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. After incubation 2.5 mL of 10% tricloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge (Centorion K24OR- 2003), for 8 min. 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 extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration.

2.3.4. Scavenging effect on DPPH radicals

The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Oyaizu, 1986). 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 left 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 in a PG Instruments Ltd. T70 UV/VIS spectrometer. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH − AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration.

2.3.5. Scavenging effect on superoxide radicals

The superoxide radical was determined by the PMS–NADH generating system, as described by Fernandes et al. (1999) with minor modifications. Briefly, 150 μL of the extract solution were mixed with 150 μL of NADH (166 μM), 450 μL of NBT (86 μM) and 150 μL of PMS (16.2 μM) (final concentrations in 900 μL; all the components of the mixture were dissolved in phosphate buffer, KH_2PO_4, 19 mM, pH 7.4). The changes of absorbance at 560 nm were recorded during 3 min at 560 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer, and the data acquisition was achieved in UV-WIN5 software V 5.0.5. Data was expressed as ΔA_{560} nm/min. The scavenging activity on superoxide radicals was calculated as follows: $((\Delta A_{560}$ nm/minblank $- \Delta A_{560}$ nm/minsample$)/\Delta A_{560}$ nm/minblank$) \times 100\%$. EC_{50} stands for the concentration of half-inhibition.

2.4. Statistical analysis

For each extraction solvent, six independent extractions were performed and in each extraction all assays were carried out in duplicate. Results are shown as solvent mean values and standard deviation. The differences between solvents in each parameter were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program. The regression analysis between total phenols contents and IC_{50} values for reducing power, scavenging activity on DPPH assay and superoxide radicals was conducted using the same statistical package.

3. Results and discussion

Antioxidant capacities of plant extracts are largely dependent of the extract composition and conditions of the test system. The measure of antioxidant capacities are influenced by many factors, which cannot accurately and quantitatively described with one single method. To avoid this in the present work the antioxidant properties of *A. unedo* leaves were measured using three different assays: the reducing power assay, the scavenging effect on DPPH radicals and the scavenging effect on superoxide radicals, and four different extraction solvents with different polarities (water, methanol, ethanol and diethyl ether). The extraction yield and total phenols content were also evaluated in order to correlate it with the antioxidant potential.

The obtained yields, as well as the extraction yield for the different tested solvents, are showed in Table 1. The solvent used for *A. unedo* leaves extract preparation showed significant different ($p < 0.05$) capacities to extract leaf compounds and probably different composition of the extracts. Extraction yield varied between 2.86% in the diethyl ether extracts (DEE), the lowest, to 32.14% in the aqueous extracts (WE), the highest.