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Arbutus unedo L. leaves as source of phytochemicals with bioactive properties

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ABSTRACT

In recent years the strawberry tree (*Arbutus unedo* L.) is being gradually replaced by other species with higher economic value. With the ultimate goal of selecting superior genotypes, the present work was initiated to study the antioxidant and antimicrobial activities, and total phenolic content in 19 different genotypes of *A. unedo* leaves from the Trás-os-Montes region of Portugal.

The genotype Bragança 1 contains higher total phenolic content (215.0 mg GAE/$g_{extract}$) whereas the Vila Boa 4 genotype shows lower total phenolic content (148.0 mg GAE/$g_{extract}$). In both methods tested to evaluate the antioxidant activity, Vila Verde and Donai displayed the highest antioxidant capacity (EC_{50} values of 0.088 and 0.090 mg/mL, respectively, for DPPH; EC_{50} values of 0.233 and 0.245 mg/mL, respectively, for reducing power assay) while Vila Boa 2 reported the lowest antioxidant potential (EC_{50} values of 0.142 and 0.378 mg/mL, respectively, in DPPH and reducing power methods). Linear negative correlations were established between the total phenol contents and the EC_{50} values for both of the antioxidant activity methods tested. Preliminary assays for antimicrobial potential showed that extracts from *A. unedo* leaves display antibacterial activity, with MIC values of 1 and 5 mg/mL for some Gram-positive and Gram-negative bacteria, respectively. Taken together, the results suggest that *A. unedo* leaves are a potential source of natural compounds with valuable bioactive properties that could be explored by the pharmaceutical, chemical and food industries.

1. Introduction

The *Arbutus unedo* L. (Ericaceae family) is a fruit tree species distributed through the Mediterranean-Atlantic area, appearing mainly in the southern Europe, northern Africa, Ireland, Palestine and Macaronesia (Canarias) (Celikel et al., 2008). In Portugal, plantations began in the southern region and subsequently spread throughout the country, including the region of Trás-os-Montes (Northeast of Portugal) (Pedro, 1994). The *A. unedo* fruits are edible but are usually consumed only after being processed. Among the processed products are alcoholic beverages like brandy and aromatic distillate. Fruits are also used to produce sweets, jams and jellies (Alarcão-e-Silva et al., 2001; Pallauf et al., 2008).

The strawberry tree is widely used in the traditional medicine. It is recognized for having diuretic, antiseptic and laxative effects and it is used to treat cardiovascular pathologies such as arterial hypertension, atherosclerosis and thrombosis (González-Tejero, 1990; Ziyyat et al., 2002; Mekhfi et al., 2006; El Haouari et al., 2007). Moreover, the leaves are used for their astringent and purgative properties and are applied in the treatment of diabetes and inflammatory conditions (Ziyyat and Boussairi, 1998; Afkir et al., 2008; Mariotto et al., 2008).

Recent studies have shown that aqueous extracts of leaves of the strawberry tree, collected in the Trás-os-Montes region, had a high antioxidant potential (Oliveira et al., 2009a). Indeed, phytochemical studies showed that leaf extracts contain several phenolic compounds, like tannins, flavonoids and phenolic glycosides, among others (Males et al., 2006; Fiorentino et al., 2007), as well as α-tocopherol (Kivçak and Mert, 2001; Oliveira et al., 2011b). Phenolic compounds are among the natural antioxidants being studied by the scientific community due to their biological properties, e.g., antioxidant and antimicrobial activities (Zhu et al., 2004; Proestos et al., 2005; Pereira et al., 2006, 2007; Sousa et al., 2006, 2008; Malheiro et al., 2011). Many of the health problems in the modern industrialized societies, such as cardiovascular diseases, cancer, diabetes, neurological diseases and atherosclerosis, appear to be related with reactive oxygen species (ROS), playing an important role in the appearance and prevalence of such diseases. A possible way to prevent and eventually decrease the occurrence of such health problems is the inclusion of foods containing natural substances with antioxidant activity in the human diet, this way providing chemical substances able to scavenge free radicals and thereby preventing the cellular oxidative stress. *A. unedo* could thus be used as a source of health promoting compounds, either in the food industry but also in the pharmaceutical

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and chemical sectors, contributing to a higher economic exploitation of this shrub. Besides the potent antioxidant capacity and possible protective effects on human health, phenols have also been correlated with anti-inflammatory, and anti-bacterial properties (Oliveira et al., 2011a). The discovery of new antimicrobial agents is an urgent need, either to fight opportunistic infections associated with the increasing number of immunocompromised individuals or the antibiotic microbial resistance, considered a serious global health problem by the World Health Organization (WHO, 2000). The presence of high levels of phenolic compounds leads to the hypothesis that antimicrobial effects could be displayed by *A. unedo*, although there are no reports concerning this bioactivity in the plant that we know of.

In the Trás-os-Montes region of Portugal, no economic importance was attributed to *A. unedo*. Due to this fact, the areas occupied by *A. unedo* plantations are being replaced by other forest species with higher economic value. Therefore, it is urgent to act in order to enhance the production, marketing and consumption of strawberry tree derived products, contributing to the species valorization, preservation and biodiversity. Thus, the goal of this work was to characterize 19 different genotypes of leaves of *A. unedo* from the Trás-os-Montes region, characterizing their antioxidant and antimicrobial properties and total phenols, in order to evaluate and to improve the potential value of this plant.

2. Experimental

2.1. Chemicals and reagents

Gallic acid, methanol, 2,2-diphenyl-1-picrylhydrazyl, iron (III) chloride, sodium chloride, and agar–agar were obtained from Sigma–Aldrich (St. Louis, USA). Sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), trichloroacetic acid, and glucose were purchased from Merck (Darmstadt, Germany). Folin–Ciocalteu's phenol reagent, sodium carbonate anhydrous, hydrochloric acid, di-sodium hydrogen phosphate dehydrate, and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Yeast extract, peptone and tryptone were obtained from Himedia (Mumbai, India). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Samples

The leaves of *A. unedo* were collected from different municipalities of the district of Bragança. Overall 19 different site samples were selected: Vinhais municipality (Vinhais 1, Vinhais 2, Vila Verde); Bragança municipality (Bragança 1, Bragança 2, Carragosa, Donai, Faílde, Fontes, Pinela 1, Pinela 2, Pinela 3, Vila Boa 1, Vila Boa 2, Vila Boa 3, Vila Boa 4), and Vimioso municipality (Argoselo, Outeiro, Vimioso).

2.3. Samples preparation and extraction conditions

For each genotype, four different samples were collected. The leaves were removed from the stem, freeze-dried and then ground. Before any analyses (total phenols determination and antioxidant activity assays), *A. unedo* leaves (2 g/sample) were extracted with 250 mL boiling water for 45 min and filtered through Whatman no. 4 paper. The aqueous extracts were frozen, lyophilized and redissolved in water to a final concentration of 20 mg/mL.

2.4. Determination of total phenols contents

Total phenolic quantifications were performed according to Singleton and Rossi (1965), with some modifications. 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 Thermo Electron Corporation Genesys 10UV spectrometer. Gallic acid was used as standard, being the results expressed in mg of gallic acid equivalents (GAE)/g of extract.

2.5. Antioxidant activity

2.5.1. Reducing power assay

The reducing power was determined according to a described procedure (Berker et al., 2007). The extract solution (1 mL) was 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 cooling, 2.5 mL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance readings indicate higher reducing power). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

2.5.2. Scavenging effect on DPPH radicals

The capacity to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano et al. (1988). The extract solution (0.3 mL) was mixed with 2.7 mL of a methanol solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min at room temperature in the dark (until stable absorbance values were obtained). The reduction of the DPPH-radical was measured by continuous monitoring of the absorption decrease at 517 nm.

DPPH scavenging effect was calculated as the percentage of DPPH discoloration using the following equation: % 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, and A_{DPPH} 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 in the solution.

2.6. Statistical analysis

All the determinations performed in total phenols content and antioxidant activity were carried out in quadruplicate and the results express mean values and standard deviations. A regression analysis, using Excel for Windows Software, was established between total phenols content of the 19 genotypes and EC_{50} values obtained in the DPPH and reducing power antioxidant assays.

2.6.1. Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 19.0 (IBM Corporation, New York, USA). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov–Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro–Wilk's test (if $n < 50$), and the Levene's tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances