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Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*

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ABSTRACT

Natural antioxidants as nutritional supplements have gained increasing importance over the last years, due to their general lower toxicity and side effects. Halophyte plants are considered an important reservoir of bioactive molecules with multiple biotechnological applications, including antioxidant. This study reports for the first time the antioxidant activity and the phenolic composition of methanol extracts of different anatomical parts of *Limonium algarvense* Erben, an endemic halophyte species of the Southwest area of the Iberian Peninsula. Antioxidant activity was determined by different assay systems, namely radical scavenging activity (RSA) on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals and on nitric oxide (NO), ferric reducing antioxidant power (FRAP), and metal chelating activity on iron and copper. The total phenolics, flavonoids, tannins, hydroxycinnamic acids, anthocyanins, flavones and flavonols are also reported, along with the phenolic composition determined by High Performance Liquid Chromatography (HPLC). In general flowers had the highest antioxidant activity, coupled with the highest levels of phenolics. Gallic acid (GA) and catechin were the main component in flowers, roots, and peduncles and in leaves there was a dominance of epigallocatechin gallate and GA. Our results suggest that *L. algarvense*, particularly its flowers, is a promising source of bioactive antioxidants with potential applications in several fields, such as the agro-food industry, namely as functional beverage.

1. Introduction

Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are produced through metabolic reactions by the mitochondrial respiratory chain (Vera-Ramirez et al., 2011). The delicate balance between positive and damaging effects of free radicals is crucial to living organisms, and when the production of ROS and/or RNS overwhelms the antioxidant defences of the organism, oxidative stress may occur, which is the underlying cause of several degenerative diseases. Thus, the use of antioxidants can thus prevent and/or reduce the severity of different oxidative stress-related diseases, for example cancer, diabetes, cardiovascular disorders and neurological ailments (Hajhashemi et al., 2010). Phenolic compounds are recognized antioxidants and free radical scavengers. Moreover, a high number of pure phenol compounds or extracts rich in those compounds have important biological activities, such as anticancer and anti-inflammatory, and could be useful in the management of the above mentioned oxidation stress-related chronic diseases (Sousa et al., 2015; Zengin et al., 2015).

Halophytes are naturally salt-tolerant plants able to grow in extreme locations characterized by high temperature and salinity conditions, such as coastal sand dunes, salt marshes, salt flats and steppes (Ksouri et al., 2010). In order to withstand environmental constraints and cope with oxidative stress, halophytes are endowed with eco-physiological mechanisms and powerful antioxidant defence systems, usually molecules displaying important biological activities (Saidana et al., 2013). Those molecules include vitamins, phenolics, polysaccharides and glycosides, displaying a vast array of activities, for example antioxidant, antimicrobial, anti-

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inflammatory, and anti-tumoural. In turn, these bioactivities can be crucial for the prevention of several diseases: e.g., cancer, chronic inflammation and cardiovascular disorders (Ksouri et al., 2012). In fact, halophytes are considered an outstanding and almost unexploited reservoir of novel functional foods and bioactive compounds (Ksouri et al., 2012).

The *Limonium* genus (Plumbaginaceae) comprises around 180 species commonly known as sea lavenders, and can be found in coastal areas from the arctic to the tropics (Whiting et al., 1998; Ali et al., 2013; Saidana et al., 2013). Different species of the *Limonium* genus are used in traditional medicine for the treatment of several ailments, including cardiovascular and inflammatory problems, bacterial infections, haemorrhage menstrual disorders, fever, arthritis and rheumatism (Aniya et al., 2002; Murray et al., 2004). Diverse bioactivities have already been documented in different *Limonium* species, such as antioxidant, antimicrobial, cytotoxic, antifungal, antitumoral, antiviral and immunomodulatory (Kandil et al., 2000; Aniya et al., 2002; Kuo et al., 2002; Mahasneh, 2002; Murray et al., 2004; Cantrell et al., 2007; Smirnova et al., 2009; Lee et al., 2011; Nostro et al., 2012; Tang et al., 2012; Saidana et al., 2013; Ali et al., 2013).

In Europe, 87 species from the *Limonium* genus have been identified from which 18 can be found in Portugal (Tutin et al., 1972; Franco, 1984). This work focused on the species *L. algarvense* Erben, which is an endemic species of the Southwest area of the Iberian Peninsula, including the Algarve (Portugal), Huelva and Cadiz (Spain). It is an obligatory halophyte found in different salinity conditions, such as coastal sand dunes and salt marshes. Despite the ethnopharmacological use of plants belonging to the *Limonium* genus, to the best of our knowledge, nothing is known about the chemical profile or biological activities of this species. In this context, this work aimed to determine the in vitro antioxidant activity of methanol extracts obtained from different anatomical parts (roots, leaves, flowers and peduncles) from *L. algarvense*. The phenolic profile of the extracts was also obtained using spectrophotometric methods and High Performance Liquid Chromatography (HPLC) analysis.

2. Materials and methods

2.1. Chemicals

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals, sodium nitrite, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), *p*-hydroxybenzoic acid, catechin, vanillic acid, cafeic acid, syringic acid, epigallocatechin gallate, coumaric acid, salicylic acid, ferulic acid and rosmarinic acid, 4-hydroxybenzaldehyde, apigenin, butylated hydroxytoluene (BHT), chlorogenic acid, epicatechin, epigallocatechin, flavone, gentisic acid, *m*-hydroxybenzoic acid, oleanolic acid, quercetin, resveratrol and *trans*-cinnamic acid, gallic acid, rutin hydra-- Ciocalteu (F–C) phenol reagent and phosphoric acid. Additional reagents and solvents were obtained from VWR International (Belgium).

2.2. Plant material

Samples from *Limonium algarvense* Erben were collected in the South of Portugal (Ludo) in June of 2013. The taxonomical classification was determined by the botanist Dr Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) and a voucher specimen was kept in the herbarium of the MarBiotech laboratory (voucher code MBH01). Plants were divided in roots, leaves, flowers and peduncles, oven dried for 3 days at 50 °C, powdered and stored at −20 °C until needed.

2.3. Extraction

Dried samples were mixed with methanol (1:40, w/v), and extracted overnight at room temperature (RT), under stirring. Extracts were filtered (Whatman n° 4) and concentrated under reduced pressure. Dried extracts were dissolved in methanol and stored at −20 °C at the concentration of 10 mg/mL.

2.4. Determination of antioxidant activity by radical-based assays

2.4.1. Radical scavenging activity (RSA) on DPPH radical

The RSA on the DPPH radical was evaluated according to Brand-Williams et al. (1995) adapted to 96-well microplates (Moreno et al., 2006). Samples (22 μL, at concentrations ranging from 0.06 to 1 mg/mL) were mixed with 200 μL of DPPH solution (120 μM) in methanol in 96-well flat bottom microtitration plates, and incubated in darkness at RT for 30 min. The absorbance was measured at 517 nm and RSA was expressed as percentage of inhibition, relative to a control, containing methanol in place of the sample and as half maximal inhibitory concentration (IC_{50}, mg/mL). Butylated hydroxytoluene (BHT, 1 mg/mL) was used as a positive control.

2.4.2. RSA on ABTS radical

The RSA on ABTS radical was evaluated by the method described by Re et al. (1999). A stock solution of $ABTS^{\bullet+}$ (7.4 mM) was generated by reacting equal amounts of ABTS with potassium persulfate (2.6 mM) for 16 h in the dark at RT. The $ABTS^{\bullet+}$ solution was diluted with ethanol to obtain an absorbance of at least 0.7 at 734 nm (Biotek Synergy 4). The samples (10 μL at concentrations between 0.125 and 1 mg/mL) were mixed in 96-well microplates with 190 μL of $ABTS^{\bullet+}$ solution. After a period of incubation of 6 min the absorbance was measured at 734 nm (Biotek Synergy 4). Results were expressed as percentage of inhibition relative to a control containing methanol and as IC_{50} values (mg/mL). BHT (1 mg/mL) was used as the positive control.

2.4.3. RSA on nitric oxide (NO)

The NO scavenging activity was evaluated according to Baliga et al. (2003). The extracts (50 μL at the concentration of 1 mg/mL) were mixed in 96 well plates with 50 μL of 10 mL sodium nitroprusside in phosphate buffer (PBS) and incubated in the light for 90 min at RT. Then, 50 μL of Griess reagent (1% of sulphanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO3 were added and absorbance were read at 546 nm (Biotek Synergy 4). Results were expressed as percentage of inhibition, relative to a control containing methanol in place of the sample. N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as the positive control at the concentration of 1 mg/mL.

2.5. Determination of antioxidant activity by metal-related methods

2.5.1. Ferric reducing antioxidant power (FRAP)

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu (1986), and modified by Megías et al. (2009). Samples (50 μL at concentrations from 0.004 to 1 mg/mL), distilled water (50 μL) and 1% potassium ferricyanide (50 μL) were mixed and incubated at 50 °C for 20 min. Then, 50 μL of 10% trichloroacetic acid (w/v) and ferric chloride solution (0.1 %, w/v) were added, and absorbance was measured at 700 nm (Biotek Synergy 4). Increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as a positive control at the concentration of