

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.

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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, caffeic 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, genticic acid, *m*-hydroxybenzoic acid, oleanolic acid, quercetin, resveratrol and *trans*-cinnamic acid, gallic acid, rutin hydrate, 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 (IC₅₀, 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^{••} (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^{••} 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^{••} 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 IC₅₀ 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 Fe³⁺ 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

1 mg/mL. Results are expressed as percentage of inhibition, relative to the positive control, and as IC₅₀ values.

2.5.2. Metal chelating activity on copper (CCA)

The CCA was determined according to Megías et al. (2009). Samples (30 µL at concentrations among 0.06 and 1 mg/mL) were mixed in 96-well microplates with 200 µL of 50 mM Na acetate buffer (pH 6), 6 µL of pyrocatechol violet (4 mM) in the above buffer and 100 µL of CuSO₄·5H₂O (50 µg/mL in water). The change in colour of the solution was measured at 632 nm using a microplate reader (Biotek Synergy 4). Results were expressed as percentage of inhibition, relative to a control, containing methanol in place of the sample and as IC₅₀ values (mg/mL). The synthetic metal chelator EDTA was used as a positive control at the concentration of 1 mg/mL.

2.5.3. Metal chelating activity on iron (ICA)

The ICA chelating activity was determined by measuring the formation of the Fe²⁺ ferrozine complex according to Megías et al. (2009), with some modifications. Samples (30 µL at the concentration of 1 mg/mL) were mixed in 96-well microplates with 200 µL of dH₂O and 30 µL of a FeCl₂ solution (0.1 mg/mL in water). After 30 min, 12.5 µL of ferrozine solution (40 mM in water) was added. Change in colour was measured in a microplate reader at 562 nm. Results were expressed as percentage of inhibition, relative to a control containing methanol, and as IC₅₀ values (mg/mL). EDTA was used as a positive control at the concentration of 1 mg/mL.

2.6. Phytochemical analysis

2.6.1. Determination of total phenolic content (TPC)

The TPC of the extracts was determined by the F–C assay according to Veloglu et al. (1998). The extracts (5 µL at the concentration of 10 mg/mL) were mixed with 10-fold diluted F–C reagent in distilled water (100 µL) and incubated at RT for 5 min. Then, 100 µL of sodium carbonate (75 g/L, w/v) were added, samples were incubated for 90 min at RT, and the absorbance measured at 725 nm on a microplate reader (Biotek Synergy 4). Results were expressed as gallic acid equivalents (GAE) in milligrams per gram of extract (dry weight, DW), using a calibration curve with gallic acid standard solutions at concentrations ranging from 0.002 to 2 mg/mL ($r^2 = 0.999$).

2.6.2. Determination of total flavonoids content (TFC)

The TFC of the extracts was estimated by the aluminium chloride (AlCl₃) colorimetric method adapted to 96-well microplates (Zou et al., 2011). The extracts (30 µL at the concentration of 10 mg/mL) were mixed in 96-well plates with 180 µL of distilled water and 10 µL of sodium nitrite (5%, w/v) and incubated for 6 min at RT. Then, 20 µL of 10% of AlCl₃ (in methanol) was added. After 6 min, 60 µL of sodium hydroxide (4%, w/v) was added and the plates further incubated for 15 min. Absorbance was measured at 510 nm in a microplate reader. A calibration curve was produced with rutin concentrations between 0.01 and 2.5 mg/mL ($r^2 = 0.9968$). Results were expressed as milligrams of rutin equivalents per gram of dried sample (mg RE/g, DW).

2.6.3. Determination of total condensed tannins content (CTC)

The CTC of the extracts was evaluated by the 4-dimethylaminocinnamaldehyde–hydrochloric acid (DMACA–HCl) colorimetric method (Li et al., 1996) adapted to 96-well microplates (Zou et al., 2011). In brief, extracts (10 µL at the concentration of 10 mg/mL) were mixed with 200 µL of a methanol solution of DMACA (1%, w/v), and 100 µL of hydrochloric acid (37%, v/v). After a 15 min incubation period, absorbance was measured at 640 nm in a microplate reader (Biotek Synergy 4). A calibration

curve was produced with catechin concentrations between 0.004 and 1 mg/mL ($r^2 = 0.9982$). Results were expressed as milligrams of catechin equivalents per gram of dried sample (mg CE/g, DW).

2.6.4. Determination of hydroxycinnamic acids and anthocyanins

Hydroxycinnamic acids and anthocyanins were estimated by spectrophotometric methods as described in Mazza et al., (1999), adapted to 96-well microplates. Briefly, extracts (20 µL at the concentration of 10 mg/mL) were mixed with 20 µL of aqueous ethanol (95%, v/v) containing 0.1% hydrochloric acid, and 160 µL of 2% hydrochloric acid was added. Absorbance was measured in a microplate reader (Biotek Synergy 4) at 320 nm to assess hydroxycinnamic acids and at 520 to evaluate anthocyanins. Calibration curves were produced with different concentrations of caffeic acid (0.002–0.5 mg/mL; $r^2 = 0.998$) and cyanidin chloride (0.004–1 mg/mL; $r^2 = 0.992$), respectively. Results were expressed as the respective standard equivalents per gram of extract (dry weight, DW) (Mazza et al., 1999).

2.6.5. Determination of flavone and flavonol content (F/F)

The F/F content was quantified according to the method described by Boulanour et al., (2013), modified to 96-well plates. Briefly, 50 µL of 2% AlCl₃–ethanol solution was added to 50 µL of the extracts at the concentration of 1 mg/mL, or standard (quercetin). After 1 h of incubation at RT, the absorbance was measured at 420 nm in a microplate reader (Biotek Synergy 4). A calibration curve was produced with quercetin concentrations between 0.002 and 0.25 mg/mL ($r^2 = 0.9937$). Results were expressed as milligrams of quercetin equivalents per gram of dried sample (mg QE/g, DW).

2.6.6. Identification and quantification of phenolic compounds by HPLC

The extracts at the concentration of 10 mg/mL in ultrapure water were analysed by HPLC–DAD (Agilent 1100 Series LC system, Germany), constituted by the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostatted column compartment (G1316A) and the diode array detector (G1315B). The data acquisition and instrumental control were performed by the software LC3D ChemStation (version Rev.A.10.02[1757], Agilent Technologies). Analyses were performed on a “Mediterranean sea 18” column, 15 × 0.21 cm, 5 µM particle size (Teknokroma, Spain). The mobile phase consists on a mixture of MeOH (solvent A) and 2.5% acetic acid aqueous solution with the following gradient: 0–5 min: 10% A, 5–10 min: 10–30% A, 10–40 min: 30–90% A, 40–45 min: 90% A, 45–55 min: 90–10% A and 55–60 min: 10% A, using a flow of 0.5 mL/min. The injection volume was 20 µL with a draw speed of 200 µL/min. The detector was set at 210, 280 (used for quantification), 320 and 350 nm. For identification, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV–visible spectral reference data. The levels of the different compounds were extrapolated from calibration standard curves. Commercial standards (gallic acid, *p*-hydroxybenzoic acid, catechin, vanillic acid, caffeic acid, syringic acid, epigallocatechin gallate, coumaric acid, salicylic acid, ferulic acid and rosmarinic acid, 4-hydroxybenzaldehyde, apigenin, BHT, chlorogenic acid, epicatechin, epigallocatechin, flavone, gentisic acid, *m*-hydroxybenzoic acid, oleanolic acid, quercetin, resveratrol, rutin hydrate, *trans*-cinnamic acid and uvaol) were prepared in methanol (10,000 mg/L) and diluted with ultrapure water to the desired concentration.

2.7. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM), and experiments were conducted at least in triplicate. Sig-

Table 1
Radical scavenging activity on DPPH, ABTS and NO radicals, metal chelating activity on iron (ICA) and copper (CCA) and ferric reducing activity (FRAP) of methanol extracts of different organs of *L. algarvense*. Results are expressed as IC₅₀ values (mg/mL).

Sample	DPPH	ABTS	NO	ICA	CCA	FRAP
Anatomical organs						
Flowers	0.09 ± 0.01 ^a	0.27 ± 0.01 ^b	>1	>1	0.29 ± 0.01	0.01 ± 0.01 ^a
Peduncles	0.37 ± 0.01 ^c	0.80 ± 0.03 ^d	>1	>1	>1	0.08 ± 0.01 ^b
Leaves	0.54 ± 0.01 ^d	0.97 ± 0.05 ^e	>1	>1	>1	0.18 ± 0.02 ^c
Roots	0.23 ± 0.01 ^b	0.60 ± 0.03 ^c	>1	>1	>1	0.06 ± 0.01 ^b
Positive controls						
BHT	0.11 ± 0.01 ^a	0.14 ± 0.01 ^a	–	–	–	–
EDTA	–	–	–	0.06 ± 0.00	0.17 ± 0.01	–
L-NAME	–	–	2.50 ± 0.01	–	–	–

Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). In the same column values followed by different letters are significantly different according to the Duncan's multiple range test ($P < 0.05$). –, not tested.

nificant differences were assessed by analysis of variance (ANOVA), or Duncan's New Multiple Range Test when parametricity of data did not prevail. SPSS statistical package for Windows (release 15.0, SPSS Inc.) was used. The IC₅₀ values were calculated by sigmoidal fitting of the data in the GraphPad Prism v. 5.0 program.

3. Results and Discussion

3.1. Antioxidant activity

Antioxidants are used to prevent several chronic disorders, including cancer, diabetes and neurodegenerative diseases, by protecting organisms from excessive ROS production. Nowadays we assist to a rising awareness of the importance to replace synthetic antioxidants by natural ones, due to their lower toxicity and side effects. In this work the antioxidant potential of different anatomical organs of the halophyte *L. algarvense* was evaluated by three radical-based assays (RSA on DPPH, ABTS and NO radicals) and by three metal-related methods (FRAP and metal chelation of iron and copper). This methodology was used because the accurate evaluation of the antioxidant capacity of a sample should be done through different complementary antioxidant assays, since oxidative stress is produced by the action of diverse reactive species presenting different mechanisms (Badarinarath et al., 2010; Niki, 2010).

Antioxidant activity varied considerably between *L. algarvense* organs (Table 1). Flowers had the highest activity towards the DPPH radical, with an IC₅₀ of 0.09 mg/mL, similar to that of the standard used, BHT (IC₅₀ = 0.11 mg/mL); roots, peduncles and leaves had also high RSA (Table 1). The extracts were less active against the ABTS•• radical, but similarly to DPPH assay the best result was also observed with flowers (IC₅₀ = 0.27 mg/mL) (Table 1). A high RSA has been previously reported for extracts made from organs of different *Limonium* species. The variability in organ-related antioxidant activity between plant organs was also observed in other halophytes, including *Mesembryanthemum edule*, *Limoniastrum monopetalum*, *Salsola kali* and *Tamarix gallica* (Ksouri et al., 2008; Falleh et al., 2012; Trabelsi et al., 2012), and is probably a result of different patterns of accumulation of secondary metabolites between different plant organs as discussed below (Del Baño et al., 2003).

All the extracts had a high capacity to reduce iron in the following order: flowers > roots > peduncles > leaves (Table 1). The capacity to reduce iron was previously reported in different extracts made from aerial organs of *L. densiflorum* (Medini et al., 2011). Molecules capable to reduce iron indicate that they are electron donors, and can therefore reduce the oxidized intermediates of lipid peroxidation processes, acting as primary and secondary antioxidants (Ordoñez et al., 2006). In this work only the flowers extract was able to chelate copper, with an IC₅₀ value of 0.29 mg/mL, and none of the samples was able to scavenge the NO radical and to

Table 2

Total content in different groups of phenolic compounds (mg/g, dry weight) in methanol extracts of flowers, peduncles, leaves and roots of *Limonium algarvense*.

Compound type	Flowers	Peduncles	Leaves	Roots
Total phenolics	228 ± 2 ^a	83 ± 4 ^c	54 ± 1 ^d	118 ± 3 ^b
Total flavonoids	236 ± 8 ^a	44 ± 2 ^c	51 ± 2 ^c	83 ± 6 ^b
Total condensed tannins	145 ± 5 ^a	19 ± 1 ^c	14 ± 0 ^c	43 ± 1 ^b
Hydroxycinnamic acids	102 ± 2 ^a	18 ± 1 ^c	41 ± 1 ^b	21 ± 1 ^c
Anthocyanins	14 ± 1 ^a	nd	18 ± 1 ^a	nd
Flavone and flavonols	23 ± 1 ^a	nd	14 ± 1 ^b	nd

Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). In the same row, values followed by the different letter are significantly different at $P < 0.05$ (Duncan's multiple range test). Total phenolics: mg GAE/g DW; total flavonoids: mg RE/g DW; total condensed tannins: mg CE/g DW; Hydroxycinnamic acid derivatives: mg CAE/g DW; Flavonols: mg QE/g DW; Anthocyanins: mg CCE/g DW; flavone and flavonols: mg QE/g DW. nd: not detected.

chelate iron. There are studies indicating that an increase in the concentration of copper ions in AD patients is related with ROS generation and neuronal cell death (Gaeta and Hider, 2005).

From our results it is clear that the methanol extract of flowers of *L. algarvense* contains molecules with both radical scavenging activity and metal reducing/chelating properties. This indicates that those molecules can act as primary antioxidants, neutralizing free radicals and preventing the initiation and propagation of oxidative chain reactions, and as secondary antioxidants, suppressing oxidative damage through the inhibition of radical formation (Loganayaki and Manian, 2010). To the best of our knowledge, there are no reports on the copper chelating capacity of species belonging to the genus *Limonium*.

3.2. Phenolic composition

Phenolic compounds are recognised antioxidant agents, and thus different patterns of accumulation of phenolics can have implications on the antioxidant activity (Foti, 2007; Dai and Mumper, 2010). In this sense, a comparative evaluation of the content of different phenolic groups of the different organs was made using spectrophotometric (colorimetric) techniques, which are widely used and convenient when dealing with samples of unknown composition (Naczek and Shahidi, 2004). This included the estimation of total phenolics, flavonoids, tannins, hydroxycinnamic acids, anthocyanins, and flavone and flavonols, and results are shown in Table 2. Natural extracts are considered rich in phenolic compounds when GAE values are higher than 20 mg/g DW (Kähkönen et al., 1999). In this sense, all organs of *L. algarvense* had markedly high levels of total phenolics, superior to other medicinal halophytes species with confirmed pharmacological properties such as *L. monopetalum*, different *Mesembryanthemum* species, *T. gallica* and *S. kali* (Ksouri et al., 2008; Falleh et al., 2009a,b; 2011; Trabelsi et al., 2012). The

Table 3
HPLC–DAD analysis of phenolic compounds contents (mg/g DW) of methanol extracts of flowers, peduncles, leaves and roots of *L. algarvense*.

Peak n ^a	RT ^b (min) ^b	Compound ^c	Flowers	Peduncles	Leaves	Roots
1	1.5	Gallic acid	3.37	3.87	1.40	2.34
2	4.7	<i>p</i> -Hydroxybenzoic acid	nd	nd	0.27	nd
3	5.3	Catechin	2.87	3.84	0.49	1.24
4	7.0	Vanillic acid	0.84	0.80	0.04	0.07
5	8.1	Caffeic acid	nd	nd	nd	0.04
6	9.7	Syringic acid	1.29	0.69	0.38	0.23
7	10.5	Epigallocatechin gallate	1.65	0.90	1.72	nd
8	11.9	Coumaric acid	0.22	0.17	0.10	0.19
9	12.7	Salicylic acid	1.89	1.24	0.44	0.51
10	13.2	Ferulic acid	0.66	0.29	0.08	0.10
11	17.1	Naringin	0.13	nd	nd	0.004
12	17.6	Rosmarinic acid	–	0.34	0.07	nd
		Total	12.8	12.1	4.9	4.72

^a Corresponding peak number in the chromatogram on Fig. 1.

^b Retention times.

^c Identified by comparison of the retention parameters with the standard controls and peak purity with the UV–vis spectral reference data. nd: not detected.

phenolics distribution between *L. algarvense* organs followed a similar trend to that found for antioxidant activities, since flowers had the highest levels of all phenolics groups, except for anthocyanins, which were equally concentrated in flowers and leaves. Having in mind that phenolics are potent in vitro antioxidants (Foti, 2007; Dai and Mumper, 2010), it is likely that the higher concentration of phenolic compounds in flowers is responsible for the higher antioxidant activity of these extracts. Similar correlations were obtained for different extracts of other *Limonium* species, such as *L. wrightii* and *L. brasiliense* (Aniya et al., 2002; Murray et al., 2004), and also for other halophytes, such as *M. edule*, *L. monoptalum* and *T. gallica* (Fallen et al., 2008; Ksouri et al., 2009; Trabelsi et al., 2012).

Besides being considered to be the most common secondary metabolites in photosynthetic organisms, phenolics are involved in many interactions between plants and the environment, against herbivory, for example, and accumulate in different plant tissues and cells due to the different biochemical/physiological roles of each organ (Hutzler et al., 1998; Bedgood et al., 2005). For example, flowers usually contain high levels of flavonoids, contributing to pigmentation (Hutzler et al., 1998; Atmani et al., 2009). Similar to the results observed in this work, a higher accumulation of phenolic compounds in flowers was already reported for other species of halophytes, such as *T. gallica* and *L. avei* (Ksouri et al., 2009; Nostro et al., 2012). Roots of *L. algarvense* had also high amounts of total phenolics (TPC: 118 mg GAE/g DW), flavonoids (TFC: 83 mg RE/g DW) and tannins (43CE mg RE/g DW), a result consistent with that observed for the methanol extract of *L. brasiliense* roots in which several polyphenolic compounds and flavonoids were identified (Murray et al., 2004). In roots, flavonoids are usually involved in defence against pathogens, in antioxidant and metal chelation of soil components, and in allelopathy (Hassan and Mathesius, 2012). Tannins make roots resistant to the microbial decomposition, and also mitigate the iron excess injury in roots (Kimura and Wada, 1989).

3.3. HPLC profile of the main phenolics present in *L. algarvense* organs

In order to gain more knowledge about the phenolic composition of the organs of *L. algarvense*, the identification of some individual phenolic compounds was performed by HPLC analysis, and results are summarized in Figs. 1 and 2 and Table 3. From the twenty-six standards tested, only twelve compounds were identified in *L. algarvense* extracts. The phenolic compounds identified in the different extracts fall into different categories: hydroxybenzoic acids (gallic, *p*-hydroxybenzoic, vanillic, syringic and salicylic acids), hydroxycinnamic acids (caffeic, coumaric, ferulic and rosmarinic acids) and flavonoids (catechin, epigallocatechin gallate

and naringin; Table 3). A higher number of phenolics was identified in flowers (Fig. 1A). Nonetheless, most of the identified compounds were present in all extracts, namely catechin and gallic, vanillic, syringic, coumaric, salicylic, and ferulic acids, although at different amounts (Table 3). The concentration of these phenolics varied considerably between the different organs, flowers and peduncles having the highest sum of identified compounds (12.8 and 12.1 mg/g DW, respectively), followed by leaves and roots (4.9 and 4.7 mg/g DW, respectively; Table 3).

Gallic acid and catechin were the principal components in flowers (3.37 and 2.87 mg g^{−1} DW, respectively) and peduncles (3.87 and 3.84 mg/g DW, respectively; Table 3). In the leaves, epigallocatechin gallate (1.72 mg/g DW) and GA (1.40 mg/g DW) were the main compounds detected (Table 3). Vanillic and coumaric acids were also identified in similar amounts in flowers and peduncles, while flowers had higher levels of epigallocatechin gallate (EGCG), syringic, salicylic and ferulic acids (Table 3). As stated above, phenolic compounds are generally produced by plants as a result of environmental interactions, and thus their distribution amongst the diverse tissues and organs of a plant is usually related to their function (Kutchan, 2001; Lattanzio et al., 2006). For example, GA was described as a pathogenic fungi inhibitor, limiting the spore germination and hyphal growth (Dix, 1979; Hättenschwiler and Vitousek, 2000). Flowers are usually rich in flavonoids that contribute to pigmentation attracting pollinators to increase the fertilization rates, and animals to eat the fruits and spread the seeds (Mol et al., 1998; Pichersky and Gang, 2000). In this sense, the presence of catechin, epigallocatechin gallate and naringin in the flowers may be explained by these functions. However, catechin and epigallocatechin gallate were also detected in considerable amounts in the peduncles (3.84 and 0.9 mg/g DW, respectively) and leaves (0.49 and 1.72 mg/g DW, respectively) extracts. Flavonoids are also produced to protect against extreme UV-radiation exposure that can disturb membranes, proteins and DNA, and lead to ROS formation (Winkel-Shirley, 2002). This type of compounds becomes even more important in halophyte plants, such as *L. algarvense*, which live in environments with high levels of irradiation; therefore they most probably act as antioxidant defence mechanism of the plants (Jithesh et al., 2006; Ksouri et al., 2010).

Other major phenol of *L. algarvense* flowers was salicylic acid (SA), and it was described to have regulatory properties in plants response to different forms of abiotic stress, namely drought, chilling, heavy metal tolerance, heat and osmotic stress (Rivas-San Vicente and Plasencia, 2011). SA also has flowering-inducing properties (Martínez et al., 2004), which may explain its presence in higher amounts in the flowers of *L. algarvense*. In spite of the similarities between the main phenolics identified in peduncles and flowers, the later organ had a higher antioxidant potential and

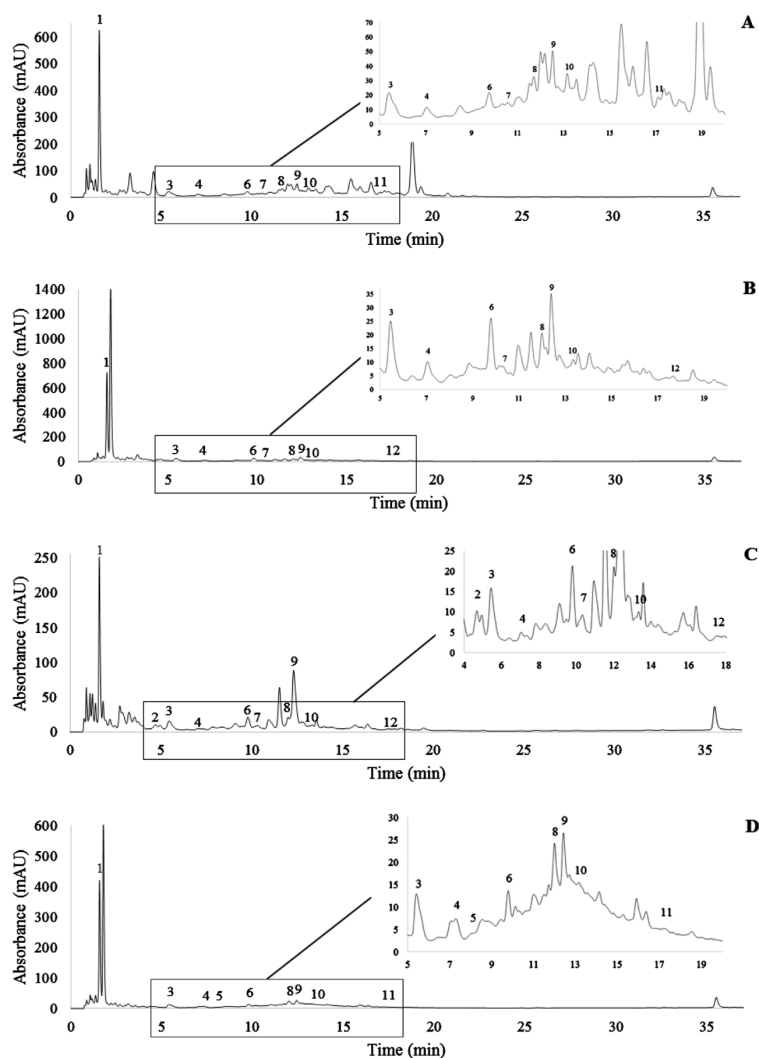


Fig. 1. HPLC–DAD analysis (280 nm) of phenolic compounds in methanol extracts of flowers (A), peduncles (B), leaves (C) and roots (D) of *L. algarvense*. The marked peaks refer to the compounds listed in Table 3.

a higher total phenolic content measured by spectrophotometric methods (Table 1). In fact, there are a few non-identified peaks in the flowers extract chromatogram (Fig. 1A), suggesting that not all the phenolic compounds present in this extract were identified by HPLC.

Some minor compounds were detected in the extracts from *L. algarvense*, namely naringin, *p*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, ferulic and rosmarinic acids. Some of those compounds were detected in specific organs, such as *p*-hydroxybenzoic acid, which was only identified in leaves (0.27 mg/g DW), and caf-

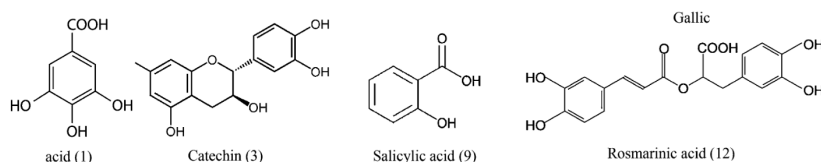


Fig. 2. Structures of some phenolic compounds detected in the methanolic extracts of flowers, peduncles, leaves and roots of *L. algarvense*. The numbers refer to the compounds listed in Table 3.

feic acid that was only detected in roots (0.04 mg/g DW). Naringin was present in flowers and roots (1.13 and 0.004 mg/g DW, respectively), and rosmarinic acid (RA) was only detected in peduncles and leaves (0.34 and 0.07 mg/g DW, respectively) (Table 3). Hydroxycinnamic acids, more specifically coumaric and ferulic acids, are associated to cell walls and have a role in plant growth, contributing to lignin biosynthesis (Lattanzio et al., 2006), which can explain their presence in all organs of *L. algarvense*. Moreover, some hydroxybenzoic acids, such as rosmarinic, caffeic and *p*-hydroxybenzoic acids, have allelopathic effects, through the inhibition of the growth of others plant species; however, this ability is not organ specific, and therefore these molecules can occur in flowers, fruits, leaves, bark, roots and exudates (Seal et al., 2004; Weir et al., 2004).

Some of the compounds detected in this study have already been reported in the *Limonium* genus. GA was previously identified in *L. wrightii* (whole plant), *L. gemellini* (roots) and *L. delicatulum* (shoots) (Anyia et al., 2002; Korulekina et al., 2004; Medini et al., 2014 Korulekina et al., 2004; Medini et al., 2014). Naringin, caffeic, ferulic and vanillic acids were previously detected in aqueous ethanol extracts of *L. avei* inflorescences (Nostro et al., 2012), while coumaric and *p*-hydroxybenzoic acids were identified in polar extracts (methanol, acetone and ethanol) of the shoots of *L. delicatulum* (Nostro et al., 2012; Medini et al., 2014). Syringic acid and EGCG were identified in *L. gemellini* roots (Korulekina et al., 2004), while in this work no epigallocatechin gallate was detected in the roots of *L. algarvense*. To the best of our knowledge this is the first report on the presence of catechin, salicylic and rosmarinic acids (Fig. 2) in the *Limonium* genus.

4. Conclusion

Our results highlight for the first time the high antioxidant activity of different organs of the Iberian endemism *L. algarvense*, which may be related to its high content in phenolic compounds. The phenolic composition is highly different between organs suggesting different roles for these compounds. The most important outcome of this work is that the highest antioxidant activity was observed in flowers and appears to be strongly related to the phenolics gallic acid, catechin, salicylic acid and epigallocatechin gallate, which were identified as the major phenolic compounds. Moreover, this is the first report on the presence of catechin, salicylic and rosmarinic acids in the *Limonium* genus. Thus, our findings suggest that *L. algarvense*, especially flowers, has a strong potential and value as source of antioxidant compounds.

Conflict of interest

The authors declare that they have no conflict of interest.

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