



In vitro antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: A comparison with green tea (*Camellia sinensis*)



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ABSTRACT

This work reports the *in vitro* antioxidant and anti-inflammatory activities and toxicity of infusions and decoctions of *Limonium algarvense* flowers, and green tea. The total contents in different phenolic groups and the quantification of individual phenolics by HPLC are also reported. *L. algarvense* and green tea had similar antioxidant properties, except for hydroxyl radical-scavenging activity, higher on green tea, and iron chelating potential, higher on *L. algarvense*. The later species also had the uppermost anti-inflammatory potential. Green tea decoction had the highest content of phenolic groups, but the infusion of *L. algarvense* had higher amounts of salicylic, gallic and coumaric acids. *L. algarvense* was not toxic, whereas green tea was toxic for S17 cells. Under our experimental conditions, infusions and decoctions of *L. algarvense* flowers had similar or higher antioxidant and anti-inflammatory properties than green tea, and thus, may be useful for alleviating symptoms associated with oxidative and inflammatory-related diseases.

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1. Introduction

Herbal medicines have long been used to treat chronic diseases such as cancer, neurodegeneration and diabetes, usually in the form of herbal teas, also called tisanes (Büyükbalci & El, 2008). Whereas herbal teas are infusions or decoctions made from herbs, spices, or other plant material in hot water, usually devoid of caffeine, 'true' teas are prepared from the leaves of the species *Camellia sinensis* (L.) Kuntze (Chan, Lim, Chong, Tan, & Wong, 2010).

The degree of oxidation of the leaves defines the type of tea: white, yellow, green, oolong, pu-erh and black tea. Green tea is the least processed, resulting from a quick drying of the fresh leaves, with minimal oxidation, which make it richer in bioactive polyphenols comparatively to more processed teas, where these compounds are degraded during the process. The consumption of tea, especially green tea, has several well-established health benefits, namely the reduction of the incidence of oxidative stress-related diseases and cardiovascular disorders, for example (Shahidi, 2000). Recent clinical trials corroborated some of the

claimed biological properties of green tea, including the prevention of prostate cancer, reduction of insulin resistance, as well as the related dyslipidaemia and protection against oxidative stress (Kumar et al., 2015).

The health benefits of 'herbal teas' and 'true teas' (including green tea) are mainly attributed to their high phenolic content, which make these beverages one of the major sources of health promoting polyphenols in our diet (Büyükbalci & El, 2008; Parr & Bolwell, 2000; Shahidi, 2000). Phenolic compounds are acknowledged to be useful in the prevention and/or symptoms relief of several health disorders, including inflammation, coronary diseases, and ailments affecting the central nervous system, including neurodegeneration (Parr & Bolwell, 2000; Sergent, Piront, Meurice, Toussaint, & Schneider, 2010).

Lately, some European medicinal plants have begun to be used as functional constituents in beverages in order to improve the human health (Gruenwald, 2009). This is the case of halophytes, such as the sea buckthorn (*Hippophae rhamnoides* L.), which is used for attenuating inflammatory disorders, pain, cough, and to aid digestion as well as a haemostatic (Gruenwald, 2009; Guliyev, Gul, & Yildirim, 2004). Also, several *Limonium* species, commonly known as sea lavenders or marsh rosemary's, are widely used in traditional medicine in the form of infusions and decoctions with

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astringent and tonic effects (Felter & Lloyd, 1898). Those beverages are traditionally used for the treatment of different health disorders, such as diarrhoea, dysentery, mucous irritations, dyspepsia, pulmonary haemorrhage, laryngitis, bronchorrhoea, catarrhal disorders, mouth and throat ulcers, gonorrhoea, leucorrhoea, anus and uterus prolapsus, and in some ophthalmic problems (Felter & Lloyd, 1898).

The halophyte *L. algarvense* Erben is an endemic halophyte found in saltmarshes of the Iberian Peninsula southwest, from the Algarve, Portugal to Huelva and Cadiz, Spain (Rodrigues et al., 2015). In our on-going studies on the presence of bioactive molecules in halophytes common in southern Portugal, we observed that methanol extracts from *L. algarvense*, particularly the flowers, had very strong antioxidant properties and high contents of bioactive phenolic compounds (Rodrigues et al., 2015). In this sense, we expand upon the latter results through the evaluation of infusions and decoctions made from flowers of *L. algarvense* as functional beverages with antioxidant and anti-inflammatory potential. For comparison purposes, infusions and decoctions of commercial green tea were also evaluated, since it is one of the most consumed teas, with recognised health promotion properties. Both samples were evaluated for antioxidant activity by six complementary assays, and for their anti-inflammatory activity against lipopolysaccharide (LPS)-stimulated macrophages. A preliminary toxicological evaluation of the samples was made *in vitro*, through the determination of toxicity against three mammalian cells, and *in vivo* against the brine shrimp *Artemia salina*. The phytochemical characterization of the extracts was performed by spectrophotometric methods, and individual phenolics were determined by high performance liquid chromatography (HPLC) analysis.

2. Material and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), catechin, epigallocatechin gallate, epicatechin, epigallocatechin, flavone, 4-hydroxybenzaldehyde, apigenin, resveratrol, quercetin, rutin hydrate, naringin, uvaol and *p*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, salicylic, ferulic, rosmarinic, chlorogenic, gentisic, oleanolic, transcinamic and gallic acids were purchased from Sigma–Aldrich (Germany). Additional reagents and solvents were obtained from VWR International (Belgium).

2.2. Plant material

Samples from *L. algarvense* were collected in different locations of the South of Portugal, namely Ludo, Vilamoura and Castro Marim, in June of 2013, and pooled together to form a composite sample. The taxonomical classification was confirmed 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). Flowers were separated, dried for 3 days at 50 °C, powdered and stored at -20 °C until needed. Dried leaves of green tea (produced in Azores, Portugal) were bought in a regional supermarket, powdered and also stored at -20 °C.

2.3. Extraction

Infusions were prepared by mixing 1 g of dried samples with 200 mL of ultrapure boiling water for five minutes. Decoctions were prepared by boiling 1 g of dried sample into 200 mL of ultrapure boiling water for five minutes. Extracts were filtered

(Whatman no. 4), freeze dried, and dissolved in ultrapure water. Aliquots were stored at -20 °C at the concentration of 10 mg/mL until needed.

2.4. Determination of total phenolics (TPC), flavonoids (TFC) and condensed tannins (CTC) contents

TPC, TFC and CTC were determined in the extracts at the concentration of 10 mg/mL and absorbance was measured in a microplate reader (Biotek Synergy 4). TPC was assessed by the F-C assay, TFC was estimated by the aluminium chloride colorimetric method adapted to 96-well microplates, and CTC was evaluated by the 4-dimethylaminocinnamaldehyde-hydrochloric acid colorimetric method adapted to 96-well microplates. Results were expressed respectively as gallic acid (GAE), rutin (RE) and catechin (CE) equivalents in milligrams per gram of extract (dry weight, DW). All methods are described in Rodrigues et al. (2015).

2.5. 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), as described before (Rodrigues et al., 2015). For identification, the retention parameters of each assay were compared with the standard controls and peak purity was assessed using UV–visible spectral reference data. Levels of the different compounds were extrapolated from calibration standard curves. Commercial standards (catechin, epigallocatechin gallate, 4-hydroxybenzaldehyde, apigenin, BHT, epicatechin, epigallocatechin, flavone, quercetin, resveratrol, rutin hydrate, uvaol, and gallic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, salicylic, ferulic, rosmarinic, chlorogenic, gentisic, *m*-hydroxybenzoic, oleanolic and transcinamic acids) were prepared in methanol (10,000 mg/L) and diluted with ultrapure water in the desired concentration.

2.6. Radical scavenging activity (RSA) on DPPH radical

Samples were tested for RSA against the DPPH radical at concentrations ranging from 0.01 to 10 mg/mL, as described previously (Rodrigues et al., 2015). BHT was used as a positive control at the same concentrations of the samples. Results were expressed as percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as half maximal inhibitory concentration (IC₅₀ values, mg/mL).

2.7. RSA on hydroxyl radical (OH•)

The hydroxyl RSA was tested on extracts at concentrations between 1 and 10 mg/mL, by the method of Kunchandy and Rao (1990), with some modifications. The reaction mixture consisted of 40 µL of 2-deoxy-D-ribose (28 mM), 200 µL of sample, 80 µL of ethylenediamine tetraacetic acid (EDTA, 1.04 mM), 200 µM of iron (III) chloride (1:1, v/v), 40 µL of hydrogen peroxide (H₂O₂, 1.0 mM) and 40 µL of ascorbic acid (1.0 mM). All the solutions were prepared in 20 mM potassium phosphate buffer (pH 7.4). After an incubation period of 1 h at 37 °C, 400 µL of thiobarbituric acid (1%) and 400 µL of trichloroacetic acid (2.8%) were added to the reaction solution, which was further incubated in a water bath at 100 °C for 20 min. After cooling, absorbance was measured at 532 nm. Catechin (0.3–10 mg/mL) was used as standard. Results were expressed as percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as IC₅₀ values (mg/mL).

2.8. RSA on superoxide radical (O_2^-)

The superoxide RSA was performed according to Robak and Gryglewski (1988), adapted to 96-well microplates. The reaction mixture consisted of 50 μ L of Tris–HCl buffer (16 mM; pH 8.0), 50 μ L of nitroblue tetrazolium (0.3 mM in Tri-HCl buffer), 50 μ L of nicotinamide adenine dinucleotide solution (0.936 mM in a solution of sodium hydroxide 5 mM), 100 μ L of the sample (at concentrations between 0.06 and 1 mg/mL) and 50 μ L of phenazine methosulfate (0.12 mM in ultrapure water). The mixture was incubated at 25 °C for 5 min, and absorbance was measured at 560 nm. Catechin was used as positive control at concentrations ranging between 0.06 and 1 mg/mL. Results were expressed as percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as IC₅₀ values (mg/mL).

2.9. Ferric reducing antioxidant power (FRAP)

The ability of the extracts to reduce Fe³⁺ was assayed by the method described by Rodrigues et al. (2015). Absorbance was measured at 700 nm (Biotek Synergy 4), and increased absorbance of the reaction mixture indicated increased reducing power. Results were expressed as a percentage relative to the positive control (BHT, 1 mg/mL), and as IC₅₀ values (mg/mL).

2.10. Metal chelating activity on iron (ICA) and copper (CCA)

ICA and CCA were tested on samples at different concentrations (0.125–10 mg/mL) as described previously (Rodrigues et al., 2015). The change in colour was measured on a microplate reader (Biotek Synergy 4). EDTA was used as the positive control at the same concentrations of the samples. Results were expressed as percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as IC₅₀ values (mg/mL).

2.11. Cell culture

The murine leukemic monocyte-macrophage cell line (RAW 264.7) and N9 cells (microglia) were obtained from Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal); the HepG2 cell line (human hepatocellular carcinoma) was kindly provided by Dr. Vera Marques, whereas S17 cells (murine bone marrow stromal) were provided by Dr. Nuno Santos (CBME, University of Algarve, Portugal). RAW 264.7 and N9 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI), while HepG2 and S17 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), both supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL)/streptomycin (50 μ g/mL), and incubated at 37 °C in humidified atmosphere with 5% CO₂.

2.12. Anti-inflammatory activity

The cell viability of the extracts (applied at concentrations ranging from 3 to 100 μ g/mL) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described elsewhere (Rodrigues et al., 2014). Nitric oxide (NO) production was evaluated using the RAW 264.7 macrophages as described by Rodrigues et al. (2014). Cells were treated with non-cytotoxic concentrations of the extracts, i.e., those that allowed cellular viability higher than 80%, in serum- and phenol-free culture medium containing 100 ng/mL of LPS, and NO content was measured by the Griess assay. A calibration curve was prepared with different concentrations (1.5–100 μ M) of sodium nitrite as standard. Results were expressed as a percentage relative

to a control containing culture medium alone, and as IC₅₀ values (μ g/mL).

2.13. Toxicological evaluation of the samples

2.13.1. Toxicity against mammalian cell lines

HepG2 and S17 cells were plated at an initial density of 5×10^3 cells/well, while N9 cells were seeded at 1×10^4 cells/well in 96-well tissue plates. Freeze-dried infusions and decoctions were dissolved directly in culture medium and applied at the concentration of 100 μ g/mL for 72 h. Cells incubated with culture medium alone were considered as negative control. Cell viability was determined by the MTT assay, and the absorbance was measured at 590 nm (Biotek Synergy 4). Results were expressed in terms of cell viability (%).

2.13.2. Brine shrimp lethality assay

Brine shrimp (*Artemia salina*) eggs were incubated in sterile artificial seawater under constant aeration for 48 h. Artificial sea water (salinity 34 g/kg; pH 8.0) consisted of 3 mg/L of sodium fluoride, 20 mg/L of strontium chloride hexahydrate, 30 mg/L of boric acid, 100 mg/L of potassium bromide, 700 mg/L of potassium chloride, 1470 mg/L of calcium chloride dihydrate, 4000 mg/L of sodium sulphate, 10,780 mg/L of magnesium chloride hexahydrate, 23,500 mg/L of sodium chloride, 20 mg/L of sodium silicate nonahydrate and 200 mg/L of sodium bicarbonate. After hatching, the active larvae (instar II/III) were collected and used for the assays (Carballo, Hernández-Inda, Pérez, & García-Grávalos, 2002). For the assays, ten larvae were placed in each well of 24-well plates containing 1 mL of artificial seawater (negative control), or the samples at the concentration of 1000 μ g/mL (diluted in artificial seawater). After 48 h at 25 °C in the dark, the number of dead individuals was assessed. Results were expressed as percentage (%) of viability.

2.14. Statistical analysis

Results were expressed as mean \pm standard error of the mean (SEM), and experiments were conducted at least in triplicate. Significant differences were assessed by analysis of variance (ANOVA) followed by Tukey HSD test. 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. Phytochemical analysis

The total contents of phenols (TPC), flavonoids (TFC) and tannins (TCT) were determined by spectrophotometric methods,

Table 1

Total contents of phenolics (TPC), flavonoids (TFC) and condensed tannins (CTC) (mg/g, dry weight) in infusions and decoctions prepared from flowers of *Limonium algarvense*, and *C. sinensis* (green tea).

Species	Extract	TPC	TFC	CTC
<i>L. algarvense</i>	Infusion	179 \pm 2 ^c	96.0 \pm 3.4 ^c	56.1 \pm 7.4 ^b
	Decoction	191 \pm 1 ^c	106 \pm 5 ^c	44.0 \pm 6.1 ^b
<i>C. sinensis</i>	Infusion	242 \pm 3 ^b	150 \pm 2 ^b	101 \pm 16 ^a
	Decoction	290 \pm 5 ^a	180 \pm 5 ^a	145 \pm 13 ^a

Values represent the mean \pm standard error of the mean (SEM) of at least three experiments each performed in triplicate ($n = 9$). In the same column, values followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test). TPC: mg GAE/g DW; TFC: mg RE/g DW; and CTC: mg CE/g DW.

and results are summarized in Table 1. The green tea decoction had the highest TPC, TFC and CTC, with values of 290, 180 and 145 mg/g DW, respectively. The green tea infusion had also high TPC (242 mg GAE/g DW), TFC (150 mg RE/g DW) and CTC (101 mg/g DW). Although lower than those obtained with green tea, the infusion and decoction from *L. algarvense* were still rich in phenolics, and no significant differences were observed for both extracts ($p > 0.05$). Phenolics comprising phenolic acids, flavonoids and tannins are widely distributed in the plant kingdom (Pandey & Rizvi, 2009), and are valuable compounds with potential application against oxidative stress-associated diseases (e.g. cancer, diabetes,

coronary and neurodegenerative illnesses) due to their potent redox properties, as reducing agents, hydrogen donors, singlet oxygen quenchers. In addition, they can act also as metal chelators (Parr & Bolwell, 2000). Moreover, there is evidence establishing an association between the consumption of polyphenolic-rich foods and/or beverages with a reduction in the risk of development of degenerative diseases related with oxidative stress (Pandey & Rizvi, 2009).

The phenolic composition of infusions and decoctions of *L. algarvense* flowers and green tea was further investigated through the identification of some individual phenolic compounds

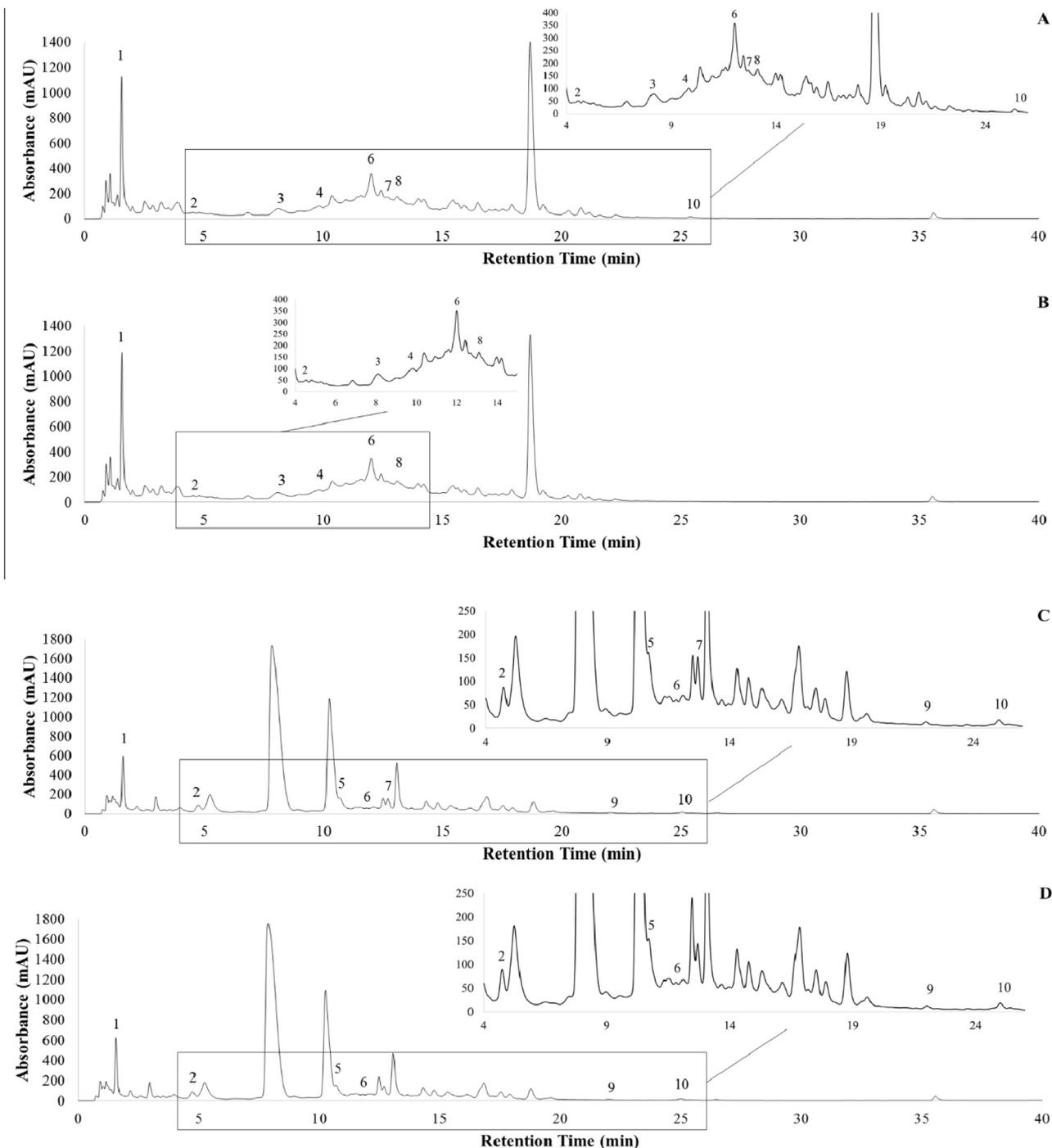


Fig. 1. HPLC-DAD analysis (280 nm) of phenolic compounds in infusions and decoctions of *L. algarvense* (A and B, respectively) and *C. sinensis* (C and D, respectively). Peak numbers refer to the compounds listed in Table 2.

Table 2
High performance liquid chromatography (HPLC–DAD) analysis of phenolic compounds content (mg/g DW) of infusions and decoctions of *L. algarvense* flowers and *C. sinensis* (green tea).

Peak no. ^a	RT (min) ^b	Compound	<i>L. algarvense</i>		<i>C. sinensis</i>	
			Infusion	Decoction	Infusion	Decoction
1	1.58	Gallic acid	5.85	6.93	4.3	4.51
2	4.72	<i>p</i> -Hydroxybenzoic acid	1.94	1.94	2.47	2.56
3	8.18	Caffeic acid	2.52	3.08	nd	nd
4	9.79	Syringic acid	2.42	2.2	nd	nd
5	10.69	Epicatechin	nd	nd	5.35	5.4
6	11.96	Coumaric acid	4.26	4.18	0.34	0.35
7	12.79	Salicylic acid	6.51	nd	7.73	nd
8	13.27	Ferulic acid	3.51	3.45	nd	nd
9	22.00	Quercetin	nd	nd	0.67	0.62
10	25.45	Apigenin	0.44	nd	0.13	0.16
		Total	27.4	21.7	20.9	13.6

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

^b Retention times. nd: not detected.

by HPLC–DAD analysis, and results are depicted in Fig. 1 and Table 2. From the twenty-six standards tested, ten compounds were identified in those samples, belonging to different families, namely hydroxybenzoic acids (gallic, syringic, salicylic and *p*-hydroxybenzoic acids), hydroxycinnamic acids (caffeic, ferulic and coumaric acids) and flavanoids (epicatechin, quercetin and apigenin) (Table 2). Among these, a total of eight compounds were detected in *L. algarvense* samples, whereas in green tea extracts seven phenolics were identified. From those, gallic, coumaric and *p*-hydroxybenzoic acids were present in all samples, but the first two in higher amounts in *L. algarvense* infusion and decoction. Besides, caffeic and syringic acids were preferentially detected in the infusion and decoction of *L. algarvense* (Table 2).

Except for salicylic acid and apigenin that were only identified in infusions (Table 2), all compounds detected were present at identical amounts in both extracts. In the infusion of *L. algarvense* the main compounds detected were salicylic, gallic and coumaric acids (6.51, 5.85 and 4.26 mg/g DW, respectively; Table 2); whereas in the decoction gallic (6.93 mg/g DW) and coumaric (4.18 mg/g DW) acids were the main compounds detected. The green tea infusion contained mainly salicylic acid (7.73 mg/g DW), epicatechin (5.35 mg/g DW) and gallic acid (4.3 mg/g DW). The presence of salicylic acid only in the infusions either of *L. algarvense* or green tea can be explained due to degradation caused by a longer exposure to high temperatures (Lindquist & Yang, 2011). The typical flavonoids from green tea, epicatechin and quercetin, were only detected in *C. sinensis* samples and not in *L. algarvense*, which sustain the use of green tea as one of the richest sources of those compounds (Chan et al., 2010). In turn, *L. algarvense* extracts were richer in hydroxybenzoic (syringic acid) and hydroxycinnamic acids (caffeic and ferulic acids) than green tea and thus, these beverages could be a potential source of those phenolic acids, which are considered one of the main components responsible for

the functional properties of different herbal preparations (Dai & Mumper, 2010). Previous studies in polar extracts from other species belonging to the *Limonium* genus have also reported the presence of gallic, caffeic, ferulic, *p*-hydroxybenzoic, coumaric and syringic acids, as well as apigenin (Aniya et al., 2002; Korul'kina et al., 2004; Medini, Fellah, Ksouri, & Abdelly, 2014; Nostro et al., 2012). Salicylic acid was also previously detected in methanol extracts of *L. algarvense* flowers, peduncles, leaves and roots, from the same site of collection (Rodrigues et al., 2015). Quercetin, coumaric and salicylic acids were formerly reported in *C. sinensis* infusions (Jeszka-Skowron & Zgoła-Grzeškowiak, 2014; López-Gutiérrez, Romero-González, Plaza-Bolaños, Martínez Vidal, & Garrido Frenich, 2015); gallic acid and epicatechin were identified in green tea aqueous extracts (Bae, Ham, Jeong, Kim, & Kim, 2015; López-Gutiérrez et al., 2015), as well apigenin and its derivatives (Forrest & Bendall, 1969; López-Gutiérrez et al., 2015).

3.2. Antioxidant activity

In anaerobic organisms, the imbalance between the antioxidant defence system and reactive oxygen species (ROS) and free radicals production, can damage cellular macromolecules (e.g. DNA, proteins and lipids) contributing to the development of pathological conditions, such as cancer, degenerative disorders and inflammation (Kohen & Nyska, 2002). Antioxidant compounds are able to stabilize or deactivate free radicals before cellular damages occur, and thus, are pivotal for the maintenance of ideal cellular and systemic conditions. In this work *L. algarvense* had in general a comparable or even higher antioxidant activity than green tea (Table 3). Regarding RSA, *L. algarvense* had the same capability to scavenge DPPH and hydroxyl radicals, but was more effective against superoxide radical (Table 3). On the other hand, *C. sinensis* samples had higher capacity to reduce iron (FRAP), but *L. algarvense* was

Table 3
Radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl (OH[•]) and superoxide (O₂^{•-}) radicals, metal-chelating activities on copper (CCA) and iron (ICA), and ferric reducing antioxidant power (FRAP) of infusions and decoctions of *L. algarvense* and *C. sinensis* (green tea). Results are expressed as IC₅₀ values (mg/mL).

Species/compounds	Extract	DPPH [•]	OH [•]	O ₂ ^{•-}	CCA	ICA	FRAP
<i>L. algarvense</i>	Infusion	0.13 ± 0.00 ^a	2.00 ± 0.23 ^{bc}	0.32 ± 0.01 ^b	0.54 ± 0.03 ^c	0.47 ± 0.01 ^b	0.17 ± 0.00 ^c
	Decoction	0.11 ± 0.00 ^a	1.82 ± 0.11 ^b	0.30 ± 0.01 ^b	0.51 ± 0.01 ^c	0.62 ± 0.00 ^b	0.17 ± 0.00 ^c
<i>C. sinensis</i>	Infusion	0.07 ± 0.00 ^a	2.42 ± 0.23 ^c	0.16 ± 0.00 ^a	0.48 ± 0.00 ^c	1.05 ± 0.05 ^c	0.13 ± 0.00 ^b
	Decoction	0.06 ± 0.00 ^a	1.71 ± 0.08 ^b	0.13 ± 0.01 ^a	0.41 ± 0.01 ^b	1.16 ± 0.06 ^c	0.12 ± 0.00 ^a
BHT [*]		0.11 ± 0.01 ^a	–	–	–	–	–
Catechin [*]		–	0.07 ± 0.00 ^a	0.62 ± 0.00 ^c	–	–	–
EDTA [*]		–	–	–	0.17 ± 0.01 ^a	0.06 ± 0.00 ^a	–

^{*} Positive controls. 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 Tukey HSD test (*p* < 0.05).

significantly ($p < 0.05$) more efficient than green tea in terms of iron chelating potential. Samples were generally less active in the copper chelation assay, and *L. algarvense* and green tea had similar copper chelation capacity. High RSA has been previously reported for extracts made from other *Limonium* species, including *L. delicatulum* (Medini et al., 2014). However, in that study, the same extract did not show any iron reducing activity at concentrations up to 1 mg/mL (Medini et al., 2014).

Superoxide (O_2^-) and hydroxyl (OH^\bullet) radicals are continuously formed within the organism, as a result of the reduction of oxygen to water in the mitochondria respiratory chain (Lipinski, 2011). In the Haber–Weiss reaction, OH^\bullet are produced by the reaction of H_2O_2 and/or O_2^- with ferric ions (Fe^{3+}) while in the Fenton reaction, ferrous ions (Fe^{2+}) react with H_2O_2 (or with hydroxyl group of water) to produce Fe^{3+} and OH^\bullet (Koppenol, 2001). Thus, scavenging those radicals and chelating the redox metals can prevent the generation of ROS, reducing the occurrence of oxidative stress-related diseases (Kohen & Nyska, 2002). From our results it is clear that infusions and decoction made from flowers of *L. algarvense* contain molecules able not only to scavenge free radicals, namely OH^\bullet and O_2^- , but also to reduce Fe^{3+} and to chelate transition metals, and thus may be useful in the prevention of oxidative-stress diseases, including coronary diseases and cancer, and age-related degenerative brain disorders (Dai & Mumper, 2010; Kohen & Nyska, 2002). Phenolic compounds have a recognised strong antioxidant capacity (Dai & Mumper, 2010). In this sense, we can suggest that the antioxidant activity of *L. algarvense* most likely reflects its high content in phenolics, especially in salicylic, gallic and coumaric acids, which are the main compounds detected. Nonetheless, all the other detected phenolic compounds may contribute to the *L. algarvense* antioxidant capacity, through additive and/or synergistic effects (Dai & Mumper, 2010). Furthermore, differences between the phenolic composition of *L. algarvense* and green tea samples can be responsible for their different behaviours against the various oxidative agents, since these compounds can have distinct activities towards the same oxidant. For instance, phenolic acids mainly present in *L. algarvense* extracts, namely gallic and caffeic acids, are excellent iron chelators, and they may be associated with the increased activity of these extracts relatively to green tea, where they are in minor amounts or absent. Gallate and dihydroxy groups, in particular, can prevent metal-induced free radicals formation through Fe^{2+} or Cu^{2+} coordination, which leads to inactive complexes formation (Dai & Mumper, 2010). In the same way, all samples and standards tested were generally less effective against the OH^\bullet radical than against other oxidants, possibly due to a differential selectivity of the antioxidants towards the several oxidising agents (Dai & Mumper, 2010; Niki & Noguchi, 2000). Taken together, our results indicate that infusions and decoctions of *L. algarvense* flowers have significant *in vitro* antioxidant properties, comparable to green tea, and hold the potential to be used as functional antioxidant herbal beverages.

3.3. Anti-inflammatory activity

For the determination of the *in vitro* anti-inflammatory activity of the samples, only those concentrations allowing cell viability higher than 80% were selected for the assay. In this case, none of the extracts exhibited cytotoxicity up to 100 μ g/mL (data not shown), and therefore all the samples were used. Stimulation of RAW 264.7 macrophages with LPS is known to induce the production of pro-inflammatory mediators, namely NO, which is associated with chronic inflammatory states (Kubes, 2000). The decrease in the NO production as a consequence of preincubation with an extract is thus frequently used to estimate its anti-inflammatory effect (Rodrigues et al., 2014). Incubation of RAW 264.7 cells with LPS, at the concentration of 100 ng/mL,

resulted in an increase of nitrite concentrations in cell supernatants from 0.3 μ M to around 16 μ M (data not shown). All samples significantly inhibited NO production, particularly infusions and decoctions of *L. algarvense* flowers with IC_{50} values of 46.3 and 48.5 μ g/mL, respectively (Table 4). Methanol extracts of *L. densiflorum* had already shown high NO inhibitory activity, using the same cellular model, which was possibly attributed to their high phenolic contents (Medini et al., 2015). Although not so effective, green tea extracts also had anti-inflammatory effects, but with IC_{50} values significantly higher ($p < 0.05$) than those of *L. algarvense* samples (infusion: 60.3 μ g/mL; decoction: 75.9 μ g/mL; Table 4). In fact, green tea has previously demonstrated ability to decrease the NO production of LPS-stimulated RAW 264.7 macrophages, with IC_{50} value 1.5-times higher than the ones obtained in this work (Forester & Lambert, 2011). This difference can be attributed to different sample origin, divergent extraction methodologies, and/or interspecific variability (Pandey & Rizvi, 2009).

Phenolic compounds have already been described as a promising alternative for the treatment of inflammatory-related diseases (Sergent et al., 2010). In addition, some of the major phenolics identified in *L. algarvense* flowers were previously reported to possess anti-inflammatory properties. For example, salicylic acid, detected in high amounts in infusions of both *L. algarvense* and green tea, is responsible for the anti-inflammatory effect of aspirin. Apigenin was also described to have NO inhibitory capacity in LPS-induced RAW 264.7 cells (Lee et al., 2007); and gallic, ferulic and coumaric acids have already showed *in vivo* anti-inflammatory activities, in rat and mice models (Kroes, van den Berg, Quarles van Ufford, van Dijk, & Labadie, 1992; Pragasan, Venkatesan, & Rasool, 2013; Zhu et al., 2014). Therefore, the presence of those compounds in *L. algarvense* extracts can contribute to their *in vitro* anti-inflammatory potential. The presence of apigenin, salicylic, coumaric and gallic acids (described above), as well as quercetin may also be responsible for the anti-inflammatory effect of the green tea samples (Comalada et al., 2005). Since *L. algarvense* was more effective than green tea in decreasing NO production, we suggest that infusion and decoction of this species is a promising source of polyphenols endowed with anti-inflammatory activity.

3.4. Toxicological evaluation

The determination of the toxicity of plant extracts in general, and herbal beverages in particular, is crucial to ascertain the safety of its consumption. The toxicological evaluation of natural compounds for pharmacological studies recommends the simultaneous assessment of cytotoxicity towards mammalian cells and brine shrimp (Carballo et al., 2002; Logarto Parra, Silva Yhebra, Guerra Sardiñas, & Iglesias Buela, 2001). Those assays were applied in this work, and results are summarized in Fig. 2. The application of infusion and decoction of *L. algarvense*, at the concentration of

Table 4

Anti-inflammatory effect of the application of infusions and decoctions of *L. algarvense* flowers and *C. sinensis* (green tea) on NO production in LPS stimulated macrophages. Results are expressed as IC_{50} values (μ g/mL).

Species	Extract	NO production
<i>L. algarvense</i>	Infusion	46.3 \pm 6.5 ^b
	Decoction	48.5 \pm 2.4 ^b
<i>C. sinensis</i>	Infusion	60.3 \pm 3.7 ^{b,c}
	Decoction	75.9 \pm 4.9 ^c
L-NAME*		29.1 \pm 2.1 ^a

* Positive control. Values represent the mean \pm standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). Values followed by different letters are significantly different according to the Tukey HSD test ($p < 0.05$).

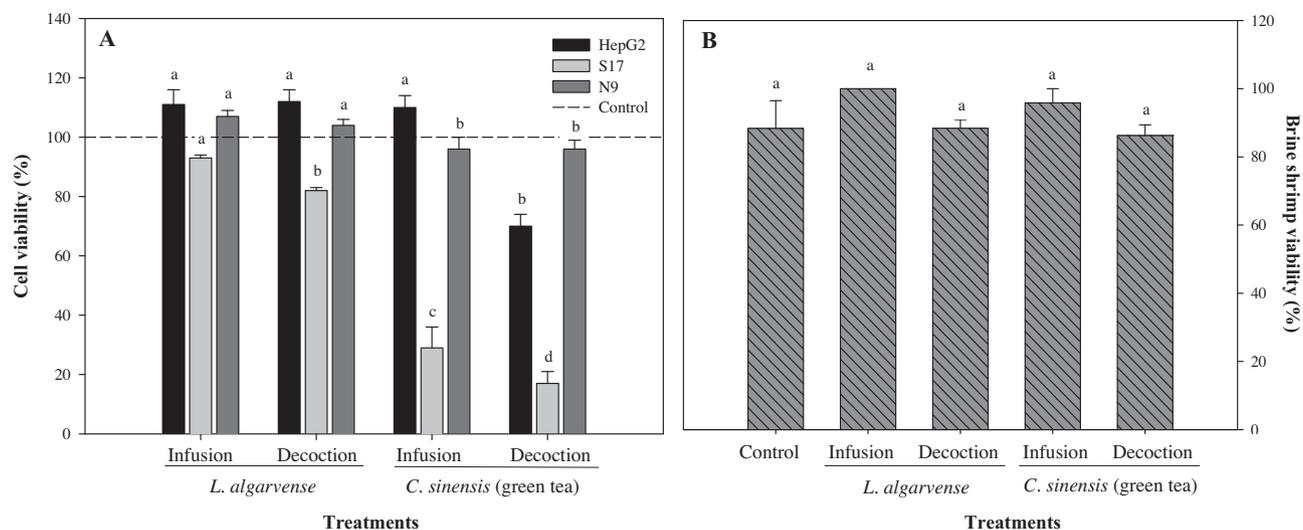


Fig. 2. Toxicity of the infusions and decoctions of *L. algarvensis* and green tea on mammalian cell lines (HepG2, S17 and N9; A), and brine shrimp (B). Cells only treated with cell culture medium (A), and brine shrimps incubated only with artificial sea water (B) were used as controls. Values represent the mean \pm standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). To the same colour/pattern of bars, different letters are significantly different according to the Tukey HSD test ($p < 0.05$).

100 $\mu\text{g}/\text{mL}$, on mammalian cell lines (HepG2, S17 and N9) resulted in values of cellular viability higher than 80% (Fig. 2A). Green tea extracts also had low toxicity on HepG2 and N9 cells ($>70\%$ of cell viability), but were toxic to the S17 cell line ($<30\%$ of cell viability). Previous studies have reported that highly concentrated green tea extracts can show some toxic effects, namely against liver and thyroid tissues (Abdel-Rahman et al., 2011). None of the extracts exerted toxic effects on the brine shrimp lethality assay at the maximal concentration of 1000 $\mu\text{g}/\text{mL}$, exhibiting a percentage of viability significantly equal to that of the artificial seawater control, and thus, can be considered non-toxic (Fig. 2B; Logarto Parra et al., 2001). The absence of toxic effects of infusion and decoction of *L. algarvensis* suggests that these aqueous extracts can be regarded as non-toxic beverages, since a positive correlation was previously established between the *in vitro* toxicity towards mammalian cell lines and the brine shrimp lethality assay, as well as towards the *in vivo* toxicity using Swiss albino mice (Carballo et al., 2002; Logarto Parra et al., 2001). However, one must keep in mind that the methods used are a preliminary toxicity screen for further experiments on mammalian animal models, which are already being pursued.

4. Conclusions

In this study, we report for the first time the *in vitro* antioxidant and anti-inflammatory activities and the phenolic composition of infusion and decoction from flowers of the halophyte *L. algarvensis*. Infusion and decoction of commercial green tea (*C. sinensis*), one of the most popular non-alcoholic beverages in the world, were also evaluated and used for comparison. *L. algarvensis* samples had similar radical scavenging activity against DPPH, hydroxyl and superoxide radicals, and also copper chelating activity than those of green tea. Nevertheless, *L. algarvensis* had higher ability to chelate iron, and to decrease nitric oxide production on LPS-stimulated macrophages, than green tea. The toxicity assessment suggest that *L. algarvensis* extracts are non-toxic. Altogether, data obtained in this work strongly suggest that infusion and decoction of the halophyte *L. algarvensis* can be considered a promising source of bioactive polyphenols able to prevent oxidative stress- and inflammation-related diseases. Assays are in progress aiming to evaluate the *in vivo* antioxidant and anti-inflammatory properties, as well as the toxicity, and the ascertainment of the bioactive molecules.

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