MARIA JOÃO DA SILVA RODRIGUES

UNRAVELLING THE BIOTECHNOLOGICAL POTENTIAL OF HALOPHYTE SPECIES FROM THE ALGARVE COAST

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Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo Ciências Biológicas

Especialidade em Biotecnologia

Trabalho efetuado sob a orientação de:

Doutora Luísa Custódio

Prof.ª Doutora Luísa Barreira

Prof. Doutor José Manuel Florêncio Nogueira

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Maria João Rodrigues)

É preciso ver o que não foi visto, ver outra vez o que se viu já, ver na Primavera o que se vira no Verão, ver de dia o que se viu de noite, com sol onde primeiramente a chuva caía, ver a seara verde, o fruto maduro, a pedra que mudou de lugar, a sombra que aqui não estava. É preciso voltar aos passos que foram dados, para os repetir, e traçar caminhos novos ao lado deles. É preciso recomeçar a viagem. Sempre.

José Saramago

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It's easier to be brave when you're not alone.

Amy Poehler

ABSTRACT

This work explored the potential of halophyte plants from the Algarve coast as sources of bioactive compounds with biotechnological applications in the pharmaceutical, food and cosmetic areas. Halophytes are salt-tolerant plants that survive in extreme environments and are equipped with powerful defence mechanisms to manage environmental stress, including the production of bioactive molecules. Despite their enormous potential, halophytes are still quite underexplored regarding their biotechnological applications. This study focused on *Frankenia laevis* L. (sea-heath), *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli, *Juncus acutus* L. (spiny rush), *J. inflexus* L. (wire rush), *J. maritimus* L. (seaside rush), *Limonium algarvense* Erben (sea lavender), and *Polygonum maritimum* L. (sea knotgrass). Methanol and dichloromethane extracts from different plant organs were prepared, evaluated for *in vitro* antioxidant, antidiabetic, anti-hyperpigmentation and neuroprotective activities, and chemically characterized.

Sea lavender and sea knotgrass were selected for their high *in vitro* antioxidant, neuroprotective, anti-hyperpigmentation and antidiabetic properties, and *Juncus* species for their *in vitro* antioxidant and neuroprotective capacities. Sea lavender and sea knotgrass had a high phenolics content, presenting a broad diversity of phenolic acids and flavonoids, respectively. The sea knotgrass had the highest *in vitro* antioxidant, anti-inflammatory, antidiabetic, neuroprotective and anti-hyperpigmentation properties, closely followed by the sea lavender. Hence, sea lavender and sea knotgrass were selected for experimental production in a greenhouse using different irrigation salinities; freshwater-irrigated plants had the best growth performance and biological properties. Juncunol, a compound isolated from the spiny rush, had *in vitro* neuroprotective properties, as well as apoptosis-inducing capacity towards hepatocellular carcinoma cells. In conclusion, the halophytes sea lavender, sea knotgrass and spiny rush can be useful sources of bioactive molecules that can potentially help to prevent oxidative stress-related diseases, delay neurodegeneration and hyperpigmentation. The spiny rush is also a promising source of compounds, namely juncunol, with *in vitro* anti-hepatocellular carcinoma activity.

Keywords: antioxidant activity, antidiabetic activity, anti-hyperpigmentation activity, anti-tumoral activity, neuroprotective activity, saline agriculture

RESUMO

Este trabalho teve como objetivo principal explorar o potencial biotecnológico de plantas halófilas do Algarve como fonte de compostos bioativos com aplicação em diferentes indústrias, tais como a alimentar, farmacêutica e cosmética. As plantas halófilas são capazes de tolerar elevadas concentrações de salinidade, e são encontradas em ambientes extremos, como sapais, salinas, dunas e praias, onde se encontram expostas a elevados níveis de radiação ultravioleta e a outros stresses ambientais, tais como secura e alagamento. De forma a minimizar os efeitos do stress ambiental, estas plantas estão equipadas com poderosos mecanismos de defesa que incluem, por exemplo, a produção de diversos compostos bioativos, como os compostos fenólicos, cujas propriedades biológicas lhes conferem variadas aplicações biotecnológicas. De facto, algumas plantas halófilas já foram utilizadas para o desenvolvimento de ingredientes inovadores para a indústria alimentar (por exemplo, *Hippophae rhamnoides* L., *Salicornia* sp. e *Chenopodium quinoa* Willd.), cosmética (particularmente *Armeria marítima* (Mill.) Willd. e *Crithmum maritimum* L.) e/ou farmacêutica (como suplementos alimentares, nomeadamente *Atriplex halimus* L. e *C. quinoa*). No entanto, tanto as atividades biológicas como o potencial biotecnológico destas plantas continuam pouco explorados. Na região do Algarve (sul de Portugal) existem várias espécies halófilas, mas poucas têm sido estudadas apesar do seu grande potencial comercial. De forma a preencher esta lacuna, este trabalho avaliou várias espécies comuns na costa Algarvia quanto ao seu potencial enquanto fontes de compostos e/ou extratos bioativos com aplicações biotecnológicas nas áreas farmacêutica, cosmética e/ou alimentar. As espécies selecionadas foram a *Frankenia laevis* L. (urze do mar), *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli, *Juncus acutus* L. (junco-agudo), *J. inflexus* L. (junco-curvado ou junco-desmedulado), *J. maritimus* L. (junco-das-esteiras ou junco-marítimo), *Limonium algarvense* Erben (ladina), e *Polygonum maritimum* L. (polígono-marítimo). Para tal, começou-se por preparar extratos de metanol e diclorometano dos diferentes órgãos (sementes, flores, pedúnculos, folhas, e/ou raízes), que foram posteriormente avaliados quanto às suas atividades antioxidante (atividade redutora de radicais e quelante de metais), antidiabética (inibição das enzimas α-glucosidase e α-amilase), anti-hiperpigmentação (inibição da enzima tirosinase) e neuroprotetora (inibição das enzimas acetil- e butirilcolinesterase) *in vitro.* A ladina e o polígono-marítimo tiveram as mais elevadas atividades antioxidante, neuroprotetora e anti-hiperpigmentação, enquanto que o polígono-marítimo revelou também elevada capacidade antidiabética. Por sua vez, as espécies de *Juncus* mostraram propriedades antioxidante e neuroprotetora. Estas espécies foram então selecionadas

para serem estudas em maior detalhe relativamente às suas propriedades biológicas e à sua composição química, usando diferentes solventes de extração, metodologias e modelos experimentais *in vitro*.

Os extratos metanólicos feitos a partir dos órgãos da ladina foram estudados quanto à sua atividade antioxidante e composição química através de cromatografia líquida de alta eficiência (HPLC). A maior capacidade antioxidante e conteúdo em compostos fenólicos, nomeadamente em catequina e ácido gálico, foi observada nos extratos das flores. Posteriormente, infusões e decocções (formulações normalmente utilizadas em medicina tradicional) das flores foram avaliadas quanto às suas atividades antioxidante, anti-inflamatória e composição química, em comparação com o chá verde, uma vez que este último é conhecido pelas suas propriedades medicinais. A ladina apresentou atividades semelhantes às do chá verde, mas menor toxicidade do que este quando testada em linhas celulares de mamífero. Os principais compostos da ladina, detetados por HPLC, foram os ácidos gálico, salicílico e gentísico. De seguida, foi estudado o potencial antioxidante, neuroprotetor e antidiabético de infusões e decocções obtidas de misturas de flores da ladina e chá verde, em diferentes proporções, para determinar possíveis efeitos sinérgicos ou antagónicos. As infusões e decocções de ambas as espécies tiveram elevada atividade antioxidante, mas a sua combinação aumentou sinergicamente a capacidade de sequestração de radicais $^{\bullet}OH$ e a redução da peroxidação lipídica. Para além disso, extratos da ladina apresentaram maior capacidade de inibição das enzimas acetil- e butirilcolinesterase, comparativamente com o chá verde, e as misturas com o chá verde tiveram efeitos sinérgicos. Por sua vez, o chá verde apresentou maior atividade inibitória da enzima α-glucosidase, mas esta atividade decresceu quando misturado com a ladina. A diversidade de compostos detetados por HPLC aumentou nas misturas, as quais ficaram enriquecidas nos ácidos hidroxibenzóico, cafeíco e siríngico. Devido aos resultados promissores obtidos com os extratos da ladina, foi testado o seu cultivo em estufa sob irrigação com água com diferentes salinidades (água doce e água salina proveniente de uma aquacultura). Avaliou-se a influência da salinidade da água da rega no crescimento das plantas, nas propriedades antioxidantes, composição química e citotoxicidade de extratos preparados a partir da biomassa dos diferentes órgãos da planta. Os resultados foram comparados com os obtidos com extratos de plantas colhidas do meio selvagem, que mostraram ser mais ativas. No entanto, as plantas cultivadas mantiveram elevada atividade antioxidante *in vitro*, especialmente as irrigadas com água doce, assim como baixa toxicidade contra células de mamífero. A análise por cromatografia líquida (LC) acoplada a espectrometria de massa de ultra-alta resolução (UHRMS/MS), mostrou que as flores possuíam maior diversidade de

compostos, e que a sua abundância variou com a salinidade da irrigação. Por exemplo, o teor de apigenina e luteolina aumentou com a salinidade, enquanto que o de miricetina diminuiu. A quercetina foi detetada apenas nas plantas silvestres, enquanto que o eriodictiol foi identificado apenas nas plantas cultivadas.

Uma vez que o género *Polygonum* é tradicionalmente utilizado para o tratamento de doenças relacionadas com a inflamação e diabetes, as propriedades antioxidante, anti-inflamatória, antidiabética foram avaliados assim como o seu perfil químico. O extrato metanólico das folhas mostrou a maior capacidade antioxidante, enquanto que o extrato de diclorometano das folhas apresentou maior atividade anti-inflamatória. O extrato de metanol das raízes e das folhas exibiu maior atividade antidiabética. A análise por cromatografia gasosa e espectrometria de massa (GC-MS) permitiu identificar os compostos β-sitosterol, estigmasterol, 1-octacosanol e ácido linolénico como os possíveis responsáveis pelas atividades observadas. Em seguida, o potencial neuroprotetor desta espécie foi estudado através da avaliação do efeito protetor *in vitro* sobre o stress oxidativo induzido e a neuroinflamação, seguido pela determinação da sua composição química por cromatografia líquida de ultra-alta pressão (UHPLC) acoplada a espectrometria de massa (MS/MS). Os extratos metanólicos (raízes e folhas) apresentaram a maior atividade neuroprotetora e anti-inflamatória, assim como de redução da citotoxicidade induzida por peróxido de hidrogénio. O polígono-marítimo foi também estudado quanto às suas propriedades anti-hiperpigmentação. Nesta fase, foram utilizados diferentes solventes e metodologias de extração, para otimizar a extração de compostos fenólicos e flavonoides. O extrato de 100% acetona foi selecionado, e apresentou elevada atividade antioxidante, anti-inflamatória e anti-hiperpigmentação. A análise dos seus constituintes foi realizada por LC-UHRMS, e as moléculas miricitrina, catequina e monogaloil-hexose foram as principais detetadas. À semelhança da ladina, o polígono-marítimo também foi produzido em estufa, irrigado com diferentes salinidades e sujeito a três colheitas (em intervalos de 6 semanas). A influência destes fatores no seu crescimento, perfil antioxidante, anti-inflamatório e químico (LC-UHRMS), de extratos preparados a partir da biomassa, foi estudada. Neste caso, os extratos de plantas irrigadas com água doce foram mais ativos que os provenientes de plantas irrigadas com água salgada artificial, especialmente na terceira colheita. Alguns compostos permitiram diferenciar entre salinidades ou colheitas, como o hiperosídeo, cuja concentração aumentou com a salinidade, e os teores de ácido cafeico 3-sulfato que diminuíram da primeira para a segunda e terceira colheitas.

O potencial antioxidante e neuroprotetor de extratos de diclorometano e metanol obtidos dos vários órgãos das espécies de *Juncus* foram também explorados. A espécie junco-agudo

apresentou os melhores resultados, particularmente o extrato de diclorometano das folhas. O composto responsável pela atividade foi isolado e identificado por HPLC como sendo o juncunol (um composto derivado do fenantreno). Esta molécula tinha sido previamente isolada das partes aéreas do junco-agudo, tendo revelado uma promissora atividade seletiva e citotóxica contras células de carcinoma hepatocelular. Deste modo, foi feito um estudo preliminar do seu modo de ação em células de hepatocarcinoma humano. Este composto mostrou promover a indução de apoptose, potencialmente relacionada com uma diminuição do potencial de membrana mitocondrial, assim como interrupção do ciclo celular na fase G0/G1.

Considerando as espécies acima mencionadas, tanto a ladina como o polígono-marítimo apresentaram um elevado teor em compostos fenólicos, no entanto, a ladina teve um teor de flavonoides mais elevado. O perfil de fenólicos mostrou elevada prevalência de ácidos fenólicos na ladina e de flavonoides no polígono-marítimo. Os extratos de polígono-marítimo apresentaram a maior capacidade antioxidante, anti-inflamatória, neuroprotetora, antidiabética e anti-hiperpigmentação *in vitro*. No entanto, a ladina mostrou atividades bastante próximas deste. A molécula juncunol, isolado do junco-agudo, apresentou propriedades neuroprotetoras, assim como capacidade de indução de apoptose e paragem do ciclo celular em células do carcinoma hepatocelular. No geral, tanto a ladina como o polígono-marítimo mostraram ser fontes de ingredientes bioativos que podem ajudar a prevenir doenças relacionadas ao stress oxidativo, hiperpigmentação, diabetes, e distúrbios neurodegenerativos. O junco-agudo tem o potencial de ser usada como fonte de antioxidantes, agentes neuroprotetores e anti-tumorais.

Em conclusão, o resultado deste trabalho demonstra que as três espécies de plantas halófilas estudadas são boas candidatas a serem utilizadas para fins comerciais: como bebidas à base de plantas (infusões/decocções da ladina), como fontes de moléculas importantes para a indústria (junco-agudo – juncunol), ou como ingredientes para a indústria cosmética (polígono-marítimo) e farmacêutica (junco-agudo – juncunol, polígono-marítimo e/ou ladina), e ainda como nutracêuticos/suplementos alimentares (junco-agudo, polígono-marítimo e/ou ladina). Adicionalmente, o perfil químico e atividades biológicas podem ajudar a explicar as suas aplicações biotecnológicas.

Palavras Chave: atividade antioxidante, atividade antidiabética, atividade anti-hiperpigmentação, atividade neuroprotetora, atividade anti-tumoral, agricultura salina

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ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	One-way analysis of variance
BHT	Butylated hydroxytoluene
BuChE	Butyrylcholinesterase
CAE	Caffeic acid equivalents
CAT	Catalase
CCA	Copper chelating activity
CCE	Cyanidin chloride equivalents
CE	Catechin equivalents
CTC	Condensed tannin content
DMACA	4-dimethylaminocinnamaldehyde
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
F-C	Folin-Ciocalteau
FDA	Food and Drug Adminstration
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GC-MS	Gas chromatography – mass spectrometry
H_2O_2	Hydrogen peroxide
HAD	Hydroxycinnamic acid derivatives
HCC	Hepatocellular carcinoma
HepG2	Human hepatocellular carcinoma cells
HPLC-DAD	High performance liquid chromatography – diode array detection
IC_{50}	Half-maximal inhibitory concentration
ICA	Iron chelating activity
LC-HRMS	Liquid chromatography – high resolution mass spectrometry
LPS	Lipopolysaccharide
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N9	Murine microglia cells
NA	Not active
NI	Not identified
NR	Not reached
NT	Not tested
NO	Nitric oxide
NK	Natural killer
$^{\bullet}OH$	Hydroxyl radical

$O_2^{\bullet-}$	Superoxide radical
QE	Quercetin equivalents
RE	Rutin equivalents
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RT	Retention times
PD	Parkinson's disease
S17	Murine bone marrow stromal cells
SD	Standard deviation
SH-SY5Y	Human neuroblastoma cells
SOD	Superoxide dismutase
T2DM	*Diabetes mellitus* type 2
TFC	Total flavonoid content
TPC	Total polyphenolic content
TYRO	Tyrosinase
UV	Ultraviolet
WHO	World Health Organization

OUTLINE OF THE THESIS

This thesis is divided into six chapters. The first chapter corresponds to a general literature review of the main subjects of the thesis, chapter 2 presents the initial screening of biological activities, chapter 3 to 5 describe the experimental work and obtained results in a research article format, and chapter 6 comprises a general discussion and conclusions of the main results and future perspectives of this study. This thesis was planned to be a collection of published or submitted research papers (chapters 3 to 5), which affords a validation of the research that supports this thesis. All presented publications were written to stand alone, thus some repetition will be found in parts of the thesis.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Medicinal plants and marine species in drug discovery

For centuries, nature has been used as a source of medicines for the treatment of a vast array of diseases. The first uses of plants in traditional medicine date back to 2600 years BC in Mesopotamia, with records of the use of more than 1000 products derived from plants (Cragg and Newman 2013). The easy access to those plants makes them an excellent source of bioactive molecules for the development of new drugs, leading to a high diversity of plant-derived compounds used in the pharmaceutic industry (Mishra and Tiwari 2011). Actually, about 25% of the drugs prescribed worldwide originate from plants (Rates 2001) and are currently used to treat 87% of all categorized human diseases including cancer and immunological disorders (Newman and Cragg 2012). For example, paclitaxel (Taxol®) and vinblastine (Velban®), derived from *Taxus brevifolia* Nutt. and *Catharanthus roseus* L., respectively, are used for cancer treatment; *Physostigma venenosum* Balf. and *Galanthus caucasicus* L. naturally produce the active principle of the commercial drugs physostigmine (Exelon®) and galantamine (Nivalin®), respectively, which are used for the treatment of Alzheimer´s disease. This worldwide trend was reinforced by the Nobel Prize laureates in physiology or medicine in 2015, where Youyou Tu was honoured by her discovery of artemisinin, a novel drug for the therapy against malaria, which was derived from *Artemisia annua* L., a plant used in the Chinese traditional medicine (The Nobel Prize in Physiology or Medicine 2015).

Recently, marine species also became a promising source of novel biologically active molecules, including microorganisms and phytoplankton, green, brown and red algae, marine invertebrates, mangroves and intertidal plants (Blunt et al. 2013). Diverse compounds were already isolated from these sources and were approved for clinical use. Ziconotide (Prialt®), was firstly isolated from the marine snail *Conus magus* and is used for severe chronic pain (Glaser and Mayer 2009). Furthermore, trabectedin (Yondelis®) used for the treatment of soft tissue sarcomas and ovarian cancer, was firstly discovered in the marine tunicate *Ecteinascidia turbinata* Herdman (Glaser and Mayer 2009). However, studies involving plants from marine environments (salt-tolerant plants – halophytes) are scarce. Although halophytes represent only 2% of terrestrial plants, they are present in about half the higher plant families with a high diversity of plant forms. And, are currently considered an important reservoir of bioactive molecules with multiple biotechnological applications (Ksouri et al. 2012).

1.2. Halophyte plants

Typically, halophyte plants are restricted to saline environments, such as salt marshes, saline arid lands, maritime dunes and marine cliffs and are exposed to extremely variable abiotic conditions, as for example salinity, light intensity, drought and temperature. Different authors use different criteria to separate halophytes (salt tolerant) and glycophytes (non-salt tolerant). Usually, halophytes are defined as highly salt tolerant plants able to complete their life cycle under salinity conditions higher than 200 mM (Flowers and Colmer 2008), or as plants able to survive in high salinity soils, with conductivity above 4 dS/m (Grigore et al. 2012). However, these limits can be restrictive regarding the wide range of salt tolerance that can vary from 25 mM for *Cicer arietinum* L. (a non-salt tolerating species) to up to 1000 mM of NaCl for *Arthrocnemum macrostachyum* (Moric.) Moris and *Tecticornia* spp., which are highly salt tolerating species (Flowers et al. 2010; Hasanuzzaman et al. 2014; Santos et al. 2015; Grigore and Toma 2017).

Regardless of the constant knowledge improvement concerning halophytes' biology, their classification is still uncertain and highly debated (Grigore et al. 2012). Irrespective of the degree of salt tolerance, halophytes can be classified into different categories concerning their ecological behaviour, distribution, response to salinity and salt intake (Aslam et al. 2011). Concerning salt requirement, they can be grouped into obligatory halophytes (also called true halophytes) that require salt to have optimal growth rates (*e.g. Suaeda maritima* (L.) Dumort. and *Mesembryanthemum crystallinum* L.); facultative halophytes that grow well with salt but can also be found in non-saline environments (*e.g. Puccinellia maritima* (Huds.) Parl. and *Thellungiella halophila*); or supporting halophytes that are capable of living in saline environments but cannot compete with local species (*e.g. Rumex maritimus* L. and *Polygonum aviculare* L.) (Aslam et al. 2011; Duarte et al. 2014; Grigore and Toma 2017). Besides, halophytes that thrive in wet conditions such as saltmarshes are classified as hydro-halophytes (*e.g. Lythrum salicaria* L. and *Plantago lagopus* L.), whereas those that live in soils with low water content (sand or cliffs) are called xero-halophytes (*e.g. Spergularia rubra* (L.) J. & C. Presl and *Medicago lupulina* L.) (Aslam et al. 2011; Öztürk et al. 2016).

The high concentration of chloride and sodium ions is extremely toxic to plants, inducing stomatal closure (leading to decreased carbon fixation), hyperosmotic shock, inhibition of photosynthesis and of cell division, nutrient imbalance, and reduction in water absorption and of plant yield (decreased cell size) (Aslam et al. 2011). To cope with the high salt concentration, halophytic species have developed diverse physiological and biochemical

adaptations, which include antioxidant enzymatic defence mechanisms, synthesis of antioxidant metabolites, and maintenance of ionic and osmotic homeostasis (Menezes-Benavente et al. 2004; Ben Amor et al. 2006; Jaleel et al. 2009; Flowers et al. 2010; Ksouri et al. 2012). Hence, halophytes can also be categorized according to their different adaptations through two main mechanisms: salt avoidance and salt tolerance (Table 1.1). Salt avoidance is a mechanism by which plants try to avoid the salt ions, preventing salt accumulation by decreasing the salt concentrations inside of the cells. As shown in Table 1.1, there are five main mechanisms of salt avoidance, which include, for example, salt excretion leading to salt accumulation on the leaf surface (Fig. 1.1A) or succulence that comprises an increase in cell size by increasing water content per unit of surface area (Fig. 1.1B) (Aslam et al. 2011; Grigore et al. 2012; Gupta and Huang 2014). On the other hand, salt-tolerant species evolved to be able to maintain cytoplasmic homeostasis through diverse mechanisms, such as accumulation or exclusion of salt ions, control of salt uptake by roots and their transport to the leaves, compartmentalization of ions, changes in the photosynthetic pathway, alterations in membranes structure, stimulation of antioxidant enzymes and plant hormones production, and biosynthesis of solutes (*e.g.* proteins, amino acids, sugars, polyols, polyamines, pigments) (Parida and Das 2005; Aslam et al. 2011).

Table 1.1. Mechanisms of halophyte adaptations to salt stress.

Mechanism		Example species	Ref.*
Salt avoidance			
Salt exclusion	Salt is excluded by filtration at the surface of the root or root membranes prevent salt penetration	*Rhizophora mangle*	[1,2]
Salt excretion	Salt is excreted through glands or bladders or cuticle located on each leaf	*Limonium algarvense, Mesembryanthemum crystallinum*, *Atriplex* sp.	[1,2]
Salt dilution	Dilution of ions in the tissue of the plant by increasing cellular water content – succulence.	*Salicornia* sp., *Sarcocornia* sp., *Carpobrotus edulis*	[1,2]
Compartmentalization of ions	Organ level - high salt accumulation in roots Cellular level- high salt accumulation in vacuoles to protect cellular enzymes	*Sueda maritima, Plantago maritima, Cakile maritima*	[1-3]
Abscission	Leaf release to avoid the toxic effects of the salt accumulation	*Aster tripolium, Plantago maritima*	[1,4]
Salt tolerance			
Osmotic adjustment	A biochemical mechanism to help plants to acclimate to dry and saline conditions	*Sesuvium portulacastrum*, *Spartina* sp., *Distichlis* sp.	[1-3]
Hormone synthesis	Abscisic acid (ABA) stress hormone hardens plants against excess salt	*Lophopyrum elongatum, Triticum aestivum, M. crystallinum*	[1-3]

* References: [1] Aslam et al. 2011; [2] Meng et al. 2018; [3] Ksouri et al. 2010; [4] Albert 1975.

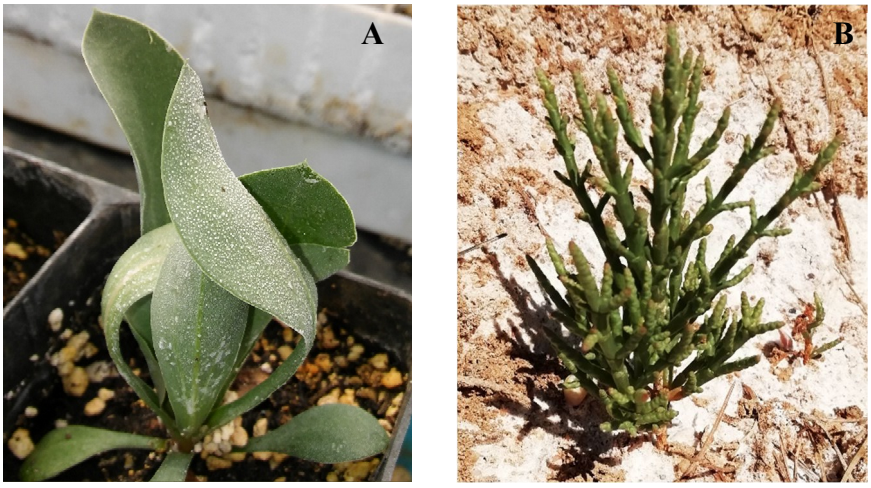

Figure 1.1. Example of halophyte adaptations to salt stress: A – Leaf salt excretion (*Limonium algarvense* Erben) and B – Succulence (*Salicornia ramosissima* J.Woods). Photos by the author.

A complete description of the existing halophytes species has not yet been made. However, in 1989, Aronson did the most extensive inventory of halophytes worldwide, reporting the occurrence of 1560 species, belonging to 540 genera and 120 families; however, it was estimated that only 20 to 30% of the halophyte species were included in that study (Aronson 1989). Considerable halophyte biodiversity occurs in southern Portugal with a manifold of potential biotechnological uses, but little research has been carried out to explore their potential. Moreover, the main value of halophytes may comprise their potential to grow in degraded lands, the possibility to be irrigated with saline water, or used as feed or forage for livestock, or as medicinal plants (Ventura et al. 2015).

1.3. Biotechnological applications of halophytes

Several halophyte species are used as medicinal and/or dietary plants, mainly in rural areas where traditional medicine is the only source of health treatments. There are some reports of the traditional medicinal uses of 43 families, endowing more than 180 halophyte species, in the Mediterranean, the Arabian Sea and Syria regions (Al-Oudat and Qadir 2011; Ksouri et al. 2012; Qasim et al. 2011), namely the Aizoaceae, Amaryllidaceae, Apiaceae, Asclepiadaceae, Asteraceae, Boraginaceae, Brassicaceae, Capparaceae, Caryophyllaceae, Chenopodiaceae, Cistaceae, Combretaceae, Convolvulaceae, Cucurbitaceae, Cyperaceae, Eleagnaceae, Euphorbiaceae, Fabaceae, Frankeniaceae, Juncaceae, Lamiaceae, Liliaceae, Malvaceae, Mimosaceae, Moraceae, Palmae, Papilionaceae, Plantaginaceae, Plumbaginaceae, Poaceae, Polygonaceae, Portulacaceae, Rhamnaceae, Rhizophoraceae, Rubiaceae, Salvadoraceae, Solanaceae, Tamaricaceae, Thymelaeaceae, Tiliaceae, Typhaceae, Verbenaceae, and Zygophyllaceae families. Within these families, several species are described to have uses in the treatment of diverse human ailments, including pain, fever and toothache, liver and digestive disorders, skin, respiratory, genito-urinary conditions, microbial and parasitic infections, inflammation, dermatitis, wounds, and burns (Al-Oudat and Qadir 2011; Ksouri et al. 2012; Qasim et al. 2011). Different plant organs, such as roots, leaves or flowers, are used for such treatments, in different forms (*e.g.* fresh or dried, raw or cooked), and applied in different ways, as for example as cataplasms, infusions, decoctions and tinctures (Al-Oudat and Qadir 2011; Ksouri et al. 2012; Qasim et al. 2011).

Botanicals always had an important role in the improvement of both human and animal health. In the particular case of halophyte plants, there are several reports of their biological activities and bioactive constituents (Table 1.2). For example, *Mesembryanthemum edule* L.,

Eryngium maritimum L. and *Artemisia campestris* subsp. *maritima* Arcang. are reported with strong *in vitro* antioxidant activities (Custódio et al. 2012; Rodrigues et al. 2014; Pereira et al. 2018, 2019), while *Tamarix gallica* L. showed *in vitro* anti-inflammatory and antimicrobial activities (Ksouri et al. 2009). *Arthrocnemum macrostachyum* and *E. maritimum* had a high *in vitro* neuroprotective and antidiabetic potential (Custódio et al. 2012; Ozarowski et al. 2015; Pereira et al. 2019), while *Juncus acutus* L. and *Beta vulgaris* subsp. *marítima* L. had *in vitro* cytotoxic activity (Rodrigues et al. 2014; Zardi-Bergaoui et al. 2017). *A. campestris* subsp. *maritima* and *Armeria pungens* Hoffmanns. & Link are described with *in vitro* anti-melanogenic properties (Pereira et al. 2018; Rodrigues et al. 2018).

Table 1.2. Examples of relevant biological activities of some halophyte species.

Biological activity	Species	Ref.*
Antioxidant	*Atriplex halimus*	[1]
	Artemisia campestris subsp. *maritima*	[2]
	Chritmum maritimum	[3]
	Erygium maritimum	[4]
	Helichrysum italicum subsp. *picardii*	[5]
	Mesembrynthemum edule	[6,7]
	Plantago coronopus	[7,8]
Anti-inflammatory	*Cynodon dactylon*	[9]
	Limonium densiflorum	[10]
	Sarcocornia fruticosa	[11]
	Tamarix gallica	[12]
Anti-microbial	*Cakile maritima*	[13,14]
	C. maritimum	[13]
	E. maritimum	[13]
	Inula crithmoides	[15]
	Limoniastrum sp.	[16,17]
	T. gallica	[12]
Neuroprotective	*Arthrocnemum macrostachyum*	[6]
	E. maritimum	[18]
	M. edule	[6]
	Pistacia lentiscus	[19]
Antidiabetic	*A. macrostachyum*	[20]
	A. maritima	[2]
	Capparis decidua	[21]
	E. maritimum	[4]
	H. picardii	[5]
	Halimione portulacoides	[20]
	Salicornia europaea	[20]
	Suaeda fruticosa	[22]
Cytotoxic	*A. halimus*	[23]
	Beta vulgaris subsp. *marítima*	[24]
	Juncus acutus	[7]
	S. fruticosa	[11]
Anti-melanogenic	*A. maritima*	[2]
	A. pungens	[25]
	P. lentiscus	[26]

* References: [1] Benhammou et al. 2009; [2] Pereira et al. 2018; [3] Pereira et al. 2017a; [4] Pereira et al. 2019; [5] Pereira et al. 2017b; [6] Custódio et al. 2012; [7] Rodrigues et al. 2014; [8] Pereira et al. 2017c; [9] Garg and Paliwal 2012; [10] Medini et al. 2017; [11] Oueslati et al. 2012; [12] Ksouri et al. 2009; [13] Meot-Duros et al. 2008; [14] Fuochi et al. 2019; [15] Jallali et al. 2014; [16] Belboukhari et al. 2005; [17] Bouzidi et al. 2016; [18] Ozarowski et al. 2015; [19] Ammari et al. 2018; [20] Zengin et al. 2018; [21] Sharma et al. 2010; [22] Benwahhoud et al. 2001; [23] Kadam et al. 2013; [24] Zardi-Bergaoui et al. 2017; [25] Rodrigues et al. 2018; [26] Lopes et al. 2016.

Moreover, several halophyte species are currently explored as sources of food and of ingredients used in cosmetic products or food supplements (Table 1.3). Several species are edible and highly procured in the food industry due to their nutritional properties, such as the case of quinoa seeds (*Chenopodium quinoa* Willd.), sea asparagus (*Salicornia* sp.) and sea fennel (*Chritmum maritimum* L.) with high commercial value and highly appreciated in gourmet cuisine (Barreira et al. 2017). Other species are currently commercialized as new fresh vegetables including *Cakile maritima* Scop. (sea rocket), *Inula crithmoides* L.*, Lampranthus sp.* (sea fingers) or *Mesembryanthemum sp.* (ice plant). Quinoa is also widely used as a food supplement to prevent hair loss, for example, while *Hippophae rhamnoides* L. (sea buckthorn) is used a source of antioxidant ingredients and *Salicornia* sp. of minerals and amino acids. The cosmetic industry also uses botanical extracts or active ingredients obtained by *in vitro* technologies from several halophyte species, as sea fennel and *Pancratium maritimum* L. for skin whitening, and *A. maritima*, *Coccoloba uvifera* (L.) L. and *Pistacia lentiscus* L. for fat elimination, and sea thrift (*Armeria maritima* (Mill.) Willd.), sea buckthorn, ice plant, sea asparagus and *E. maritimum* for skin purifying, moisturizing and anti-ageing effects (Phytomer 2019; Paiskincare 2019; Vitaminplus 2019; Riafresh 2019; QuinoaPortuguesa 2019; Presençadeluxo 2019; Seppic 2019a,b; Biotona 2019; Pipingrock 2019; Dr.hauschka 2019; Nutriherbs 2019; Optimah 2019).

Table 1.3. Examples of halophyte species with applications in the food, pharmaceutical and cosmetic areas, and respective properties and uses.

Species	Plant part	Application	Properties/Uses	Ref.*
Armeria maritima	Ns	Cosmetic Ingredient	Purifying, protect skin against pollution	[1]
Artemisia maritima	Ns	Cosmetic Ingredient	Delay fat storage, fat elimination	[1]
Aster tripolium	Ns	Cosmetic	Reduce the appearance of redness (inflammation)	[2]
Atriplex halimus	Ns	Food Supplement	Reduce menopause symptoms	[3]
Cakile maritima	Leaves	Food	Flavouring, young leaves in salads, older leaves can be mixed with milder tasting leaves and used as a potherb, the dried roots can be ground and mixed with cereal flours to make bread	[4]
Chenopodium quinoa	Seeds	Food	As a side-dish, in salads	[5]
		Food Supplement	Prevent hair loss	[6]
Crithmum maritimum	Oil	Cosmetic Ingredient	Anti-wrinkles, illuminate the complexion	[1]
	Flowers and leaves	Food	Salads, aromatic sauces, fried or preserved in vinegar, in cream, risotto, soups, vegetable rustic pies, casseroles, sauces for seasoning	[4]
	Stem cells	Cosmetic Ingredient	Whitening effect, reduce hyperpigmentation, anti-wrinkles, moisturising	[7]
Coccoloba uvifera	Ns	Cosmetic Ingredient	Reduce fat synthesis and storage, improve firmness and minimize cellulitis	[1]
Eryngium maritimum	Stem cells	Cosmetic Ingredient	Anti-ageing, improves the firmness and skin renewal	[8]
Hippophae rhamnoides	Fruit juice	Food supplement	Antioxidant	[9]
	Seed oil	Cosmetic Ingredient	Rejuvenating, moisturizing	[10]
Inula crithmoides	Leaves	Food	Seasoning for meats, fish and shellfish or in the preparation of warm salads	[4]
Lampranthus sp.	Leaves	Food	Highlight the flavour of some dishes, combining well with meats, fish and seafood or preparing warm salads, interesting when combined with mushrooms	[4]
Mesembryanthemum sp.	Flowers, leaves and stems	Food	Leaves and stems can be eaten raw or cooked, as a spinach substitute, in pickles, or as a garnish	[4]
Mesembryanthemum crystallinum	Ns	Cosmetic Ingredient	Soothing and moisturising	[11]

Pancratium maritimum	Ns	Cosmetic Ingredient	Inhibit melanin synthesis, correct the appearance of mottled skin	[1]
Pistacia lentiscus	Aromatic resin	Cosmetic Ingredient	Fat elimination, skin firming	[1]
Plantago coronopus	Leaves	Food	Young leaves can be eaten raw or cooked, in salads	[4]
Salicornia sp.	Ns	Food Supplement	Source of minerals and amino acids	[12]
	Oil	Cosmetic Ingredient	Moisturising, smoothing	[1]
	Leaves	Food	Salads, with fish, sushi, seafood, meat, pasta, as a seasoning and in drinks	[4]
Salvadora persica	Bark	Toothpaste	Oral and dental care	[13]
Sarcocornia sp.	Leaves	Food	Fresh, in salads or soups, or sauces	[4]
Schinus terebinthifolius	Seeds	Cosmetic Ingredient	Fat elimination	[1]
Sueda maritima	Leaves	Food	Seasoning for eggs, fish or shellfish, pickles	[4]
Ulex europaeus	Oil	Cosmetic Ingredient	Cellular renewal and cohesion, protection from premature skin ageing	[1]

Ns: non specified.

* References: [1] Phytomer 2019; [2] Paiskincare 2019; [3] Vitaminplus 2019; [4] Riafresh 2019; [5] QuinoaPortuguesa 2019; [6] Presençadeluxo 2019; [7] Seppic 2019a; [8] Seppic 2019b; [9] Biotona 2019; [10] Pipingrock 2019; [11] Dr.hauschka 2019; [12] Nutriherbs 2019; [13] Optimah 2019.

1.4. Halophytes cultivation

Most of the crop and forage species used in modern agriculture are salt sensitive (glycophytes) and can handle only a very limited concentration of salt in their growth media: a 10% yield decrease is observed as the soil salinity increases over the 4-8 dS/m range, although some species are much more sensitive (Panta et al. 2014). Conversely, the growth of several salt-tolerant plants (halophytes) can be stimulated within a salinity range of 15-25 dS/m (Panta et al. 2014). Therefore, halophytes can grow in conditions where conventional glycophyte crops cannot, and can be cultivated by different production systems, such as in salt-affected soils and using diverse saltwater resources, including seawater, salt-contaminated phreatic sheets, brackish water (*e.g.* from estuaries), and aquaculture effluents (Ladeiro 2012; Ventura et al. 2015). This particular feature of halophyte plants is especially relevant in the current context of climate change and soil and water salinization. In fact, nearly 7% of the earth's land surface has salt-affected soils, and those affected by sodium are even more common (Flowers et al. 1997). Irrigated land represents only 15% of the total cultivated land but produces one-third of the world's food (FAO 2012). Moreover, about 11% of the world's irrigated areas are already affected by some degree of salinization (GRID-Arendal 2018). Globally, it is estimated that every minute 3 ha of currently arable land becomes unproductive due to secondary-induced salinization (Panta et al. 2014; Custódio et al. 2017), and each year between 10 and 20 Mha of irrigated land deteriorates to zero productivity (Custódio et al. 2017). Thus, to combat these issues and to meet the needs of the growing population, it is necessary to find alternative crops species, such as halophyte plants, able to cope with these adverse salinity conditions.

As detailed in section **1.3**, numerous halophytes can be explored as innovative crops with uses in different industrial sectors. In addition, their cultivation could be an approach for the rehabilitation of saline areas, for revegetation, soil bio-remediation, mitigation of aquaculture wastes or even for ecosystem-engineering purposes (Buhmann and Papenbrock 2013; Flowers et al. 2010; Grigore and Toma 2017; Hasanuzzaman et al. 2014; Ksouri et al. 2012; Santos et al. 2015; Ventura and Sagi 2013). However, despite the recent interest on the commercial potential of halophytes (Ksouri et al. 2012; Petropoulos et al. 2018a,b), knowledge about their cultivation is scarce and there is a need to develop agronomic systems for target species that are economically, socially and environmentally feasible. In the last 30 years, several authors reported the tentative cultivation of more than 80 halophyte species, by diverse cultivation methodologies, irrigations techniques and harvest regimes (Table 1.4). For instance, the species *Atriplex* sp., *I. crithmoides* and *Salvadora persica* L. were irrigated with different

NaCl concentrations, including non-saline conditions (Gallanger 1985, Sykes and Wilson 1989, Zurayk and Baakbaki 1996, Dagar et al. 2004, Koyro 2006, Ksouri et al. 2007, Glenn et al. 2012, Peralstein et al. 2012, Belkheiri and Mulas 2013, Benzarti et al. 2014, Slama et al. 2015, Bendaly et al. 2016), while *Salicornia bigelovii* Torr. and *S. persica* were irritated with seawater in a coastal desert and salt-affected soil (Glenn et al. 1991; Rao et al. 2004). Moreover, some species, as for example *Salicornia persica* Akhani, *Tripolium pannonicum* (Jacq.) Dobrocz., *Plantago coronopus* L., *S. dolichostachya* Moss*, Sesuvium portulacatrum* (L.) L. and *Batis maritima* L. were successfully farmed via integrated multi-trophic aquaculture (IMTA) systems, where the by-products (wastes) from one or more marine organisms' species (*e.g.* fish) are recycled to become fertilizers for plant cultivation (Boestfleisch et al. 2014; Waller et al. 2015; Boxman et al. 2018).

In addition, the growth, development and chemical/functional profiles of obtained biomass may be influenced by agrotechnical practices, as for example, quantity and salinity of irrigation water, fertilization, type of soil, temperature, sunlight, the season of cultivation, time and harvest cycle. Thus, the presence and quantity of the bioactive elements with medicinal properties can be manipulated to obtain highly bioactive ingredients (Ventura et al. 2010, 2011, 2013; Boestfleisch et al. 2014). However, studies involving the influence of different saline cultivation conditions on the biochemical properties of produced plants are scarce. Some authors evaluated the influence of cultivation techniques on the nutritional profile of edible halophytes, including for example *S. bigelovii, I. crithmoides, Kosteletzkya virginica* K. Presl ex Gray*, P. coronopus, S. persica, Atriplex halimus* L.*, Sarcocornia fructicosa* (L.) A.J.Scott and *Distichlis palmeri* (Vasey) Fassett ex I. M. Johnst. (Glenn et al. 1991; Zurayk and Baalbaki 1996; He et al. 2003; Koyro 2006; Peralstein et al. 2012; Diaz et al. 2013; Shpigel et al. 2013; Benzarti et al. 2014; Bendaly et al. 2016). There are also some reports of the influence of the culture conditions on the antioxidant capacity of extracts obtained from different species, such as *C. maritima, Salicornia* sp, *T. pannonicum, Atriplex* sp., *P. coronopus, Lepidium latifolium* L.*, Bruguiera cylindrica* (Linnaeus) Blume and *S. portulacastrum* (Ksouri et al. 2007; Boestfleisch et al. 2014; Slama et al. 2015; Bendaly et al. 2016; Slama et al. 2017).

Table 1.4. List of authors and halophyte species cultivated for commercial purposes and the respective cultivation methods, treatments, harvest and parameters tested.

Author	Species	Cultivation methods	Treatments	Harvest regime	Evaluated parameters
Gallagher 1985	*Atriplex triangularis, Distichlis spicata, Sporobolus virginicus, Spartina alterniflora, Spartina patens*	Growth chamber, greenhouse and field	Different salinities (10 – 80 ‰)	Single harvest (10 months) at different months and repeated harvesting (2 months interval)	Productivity
Sykes and Wilson 1989	*Acaena anserinifolia, Ammophila arenaria, Austrofestuca littoralis, Bromus diandrus, Carex pumila, Centella uniflora, Colobanthus muelleri, Coprosma acerosa, Craspedia uniflora, Cyperus ustulatus, Desmoschoenus spiralis, Elymus fartus, Ganaphalium audax, Gnaphalium luteo-album, Gunnera albocarpa, Holcus lanatus, Hydrocotyle novae-zelandiae, Lachnagrostis lyallii, Lagenifera pumila, Lagurus ovatus, Lupinus arboreus, Phormium tenax, Plantago triandra, Poa pusilla, Scirpoides nodosa, Senecio elegans, Silene gallica, Triticum aestivum, Wahlenbergia congesta*	Glasshouse (temperature: 10 - 34ºC; humidity: 50 - 100%)	Different salinities (0, 0.25, 0.5, 0.75, 1, 1.5, 2% of NaCl)	7 and 32 days	Growth
Glenn et al. 1991	*Salicornia bigelovii*	Field plantation (coastal desert)	Seawater irrigation	220 days	Growth, oil, protein, fat, ash contents and fatty acids profile

Zurayk and Baalbaki 1996	*Inula crithmoides*	Ventilated glasshouse (spring - maximum temperature: 30°C; summer (maximum temperature 45°C)	Different salinities (0.5 (tap water), 10, 20, 40, and 80 dS/m)	3 months	Growth and nutritional value
He et al. 2003	*Kosteletzkya virginica*	Field plantation (January and July: 20.8 and 26.8ºC, respectively) and salinity among 0.1 - 1.2%	Individuals randomly selected and individuals selected on seed yield	1 year	Growth, seed production and seed quality, seed oil and crude protein contents
Dagar et al. 2004	*Salvadora persica*	Pothouse	Different salinities (0.4, 4, 8, 12, 16 dS/m)	1 year	Growth performance
Rao et l. 2004	*Salvadora persica*	Field plantation (salt-affected black soil)	Different salinities (25–35, 35–45, 45–55 and 55–65 dS/m), and irrigation with saline water (35 dS/m)	4 years	Growth, seed and oil yields
Koyro 2006	*Plantago coronopus*	Gravel/hydroponics culture (16 h light/8 h dark) in a greenhouse (temperature 15-25ºC; relative humidity 45 - 70%; irradiation intensity 100 $\mu E/m^2/s$	Different salinities (0, 125, 250, 375 and 500 mM of NaCl)	6 weeks	Growth, carbohydrates and pigments contents
Ksouri et al. 2007	*Cakile maritima*	Glasshouse (16/8 h light/darkness, 20 - 25ºC temperature, 70 - 80% relative humidity; irradiation intensity 400 $\mu mol/m^2/s$	Seeds from different sites (Jerba and Tabarka) and different salinities in culture media (0, 100 and 400 mM of NaCl)	28 days	Growth, total phenolics and antioxidant activity
Ventura et al. 2011	*Salicornia persica, Sarcocornia fructicosa*	Hydroponics	Two ecotypes with different concentrations of seawater (0, 25, 50, 75 and 100%)	2, 3 and 4 weeks harvest intervals over 5 months	Growth, ions contents, fatty acids profile, total phenolics, β-carotene, total ureides and proteins

Glenn et al. 2012	*Atriplex hortensis, A. canescens, A. lentiformis*	Greenhouse	Different salinities (0, 5, 10, 15, 20, 25, 30, 35 g/L of NaCl)	30 days	Biomass production
Grigore et al. 2012	*Inula crithmoides, Plantago crassifolia, Medicago marina*	Greenhouse with tap water irrigation	Different substrates (commercial peat, garden soil, saline soil and sand from a littoral dune)	12 weeks	Growth
Pearlstein et al. 2012	*Distichlis palmeri*	Greenhouse under anaerobic (flooded) conditions	Different salinities (0, 5, 10, 15, 20, 25, 30 g/L of NaCl)	26 days	Biomass production and yields, proximate composition
Belkheiri and Mulas 2013	*Atriplex halimus, A. nummularia*	Greenhouse (photoperiod of 14 - 15 h)	Different salinities (0, 300, 600, 800 and 1000 mM NaCl and 0, 300, 600, 800 and 1000 mM KCl for *A. nummularia*; and 0, 600 and 1000 mM NaCl and 0, 600 and 1000 mM KCl for *A. halimus*), and different irrigation regimes	10 and 20 days	Growth, ions and proline contents
Diaz et al. 2013	*Salicornia bigelovii, Atriplex lentiformis, Distichlis spicata, Spartina gracilis, Allenrolfea occidentalis and Bassia hyssopifolia*	Field experiment over a 6-year period	Saline drainage water	3 harvests in fall and summer	Biomass production and mineral content
Shpigel et al. 2013	*Salicornia persica*	Greenhouse with recirculation aquaculture system (RAS)	Aquaculture effluents from sea bream (*Sparus aurata*)	3 weeks	Growth, ash, protein, lipids and carbohydrates contents
Boestfleisch et al. 2014	*Salicornia europaea, S. dolichostachya, Tripolium pannonicum, Suaeda maritima, Atriplex portulacoides, Atriplex halimus, Plantago coronopus, Lepidium latifolium, Bruguiera cylindrica*	Greenhouse	Different salinities (0, 220, 331 and 442 mM of NaCl)	7 weeks	Growth, total phenolics and flavonoids and antioxidant activity

Benzarti et al. 2014	*Atriplex portulacoides (syn. Halimione portulacoides)*	Greenhouse (400 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR), 25°C temperature, and 60% relative humidity)	Different salinities (0, 200, 400, 800, and 1000 mM of NaCl)	40 days	Growth, ions, proline, free amino acids, total soluble sugars and glycine betaine contents
Slama et al. 2015	*Sesuvium portulacastrum*	Greenhouse (25 - 15°C (day/night) temperature, 65 - 85 (day/night) relative humidity, and 14/10 h light/dark regime)	Different field capacity (100 and 25%) and salinities (0 and 200 mM of NaCl)	1 month	Growth, polyphenols and anthocyanins contents, and antioxidant activity
Waller et al. 2015	*Tripolium pannonicum, Plantago coronopus, Salicornia dolichostachya*	Hydroponic cultures integrated into a RAS water (*Dicentrarchus labrax* L.) at 16 PSU of salinity		35 days	Biomass production and microbiological quality
Bendaly et al. 2016	*Atriplex halimus*	Glasshouse (400 $mmolm^2$ s^2 light intensity, 25ºC temperature and 70% relative humidity)	Different salinities (0, 50,100,150, 200 and 400 mM of NaCl).	2 months	Growth, amino acids, soluble sugars, polyalcohol, organic acids, minerals, pigments, antioxidant activity and total phenolics
Panta et al. 2016	*Atriplex lentiformis, Medicago arborea*	Glasshouse	Different soils (sandy loam, clay soil and a mixture of sandy loam/clay) and different salinities (0.04 dS/m, 8 dS/m, 16 dS/m)	5 months	Biomass production and physiological parameters
Radulovich et al. 2017	*Avicennia germinans, Rizophora mangle, Sesuvium portulacastrum, Sporobolos virginicus*	Floating at the sea	Seawater	733 days	Growth

Slama et al. 2017	*Sesuvium portulacastrum*	Greenhouse (25/15°C day/night temperature, 65/85% (day/night) relative humidity, and 14/10 h light/dark regime)	Different salinities (0, 200, 400, 600, 800 mM of NaCl)	1 month	Growth, polyphenols, anthocyanins, carotenoids contents and antioxidant activity
Boxman et al. 2018	*Sesuvium portulacastrum, Batis maritima*	Fully recirculating aquaponic system from aquaculture of *Sciaenops ocellatus* (redfish)		9 months	Biomass production

1.5. Biological activities

Biological activity is defined as the capacity of a substance to exert a chemical or physiological effect on cellular functions of the organism, and it is determined by the concentration and the time of exposure. The evaluation of the biological properties of botanical samples have a main application in the identification of extracts and/or compounds to be used with medicinal or cosmetic purposes. These determinations are usually performed through *in vitro* bioassays using specific biological targets according to the biological effect under study, allowing to determine the potency of a certain extract and/or compound on that particular target (Jackson et al. 2007). Despite the scientific research and commercial interests focus on terrestrial and marine natural products, edible or medicinal halophytes have not been subjected to a systematic screening of biological activities aiming its biotechnological exploitation. In particular, studies targeting halophytes present in the Portuguese coast are particularly scarce.

1.5.1. Antioxidant activity

In aerobic organisms, free radicals, reactive oxygen (ROS) and nitrogen (RNS) species are by-products from numerous physiological and biochemical processes, such as mitochondria respiratory chain reaction, microbial infections or apoptosis. During the biological reduction of molecular oxygen to water, superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and organic peroxides are produced (Fig. 1.2) (Lopaczynski and Zeisel 2001; Khan 2002; Reuter et al. 2010; Lipinski 2011; Vera-Ramirez et al. 2011). Superoxide ($O_2^{\bullet-}$) and hydroxyl ($^{\bullet}OH$) radicals are continuously formed within the organism, through the Haber-Weiss reaction, where $OH^{\bullet}$ is produced by the reaction of H_2O_2 and/or $O_2^{\bullet-}$ with ferric ions (Fe^{3+}) while in the Fenton reaction, ferrous ions (Fe^{2+}) react with H_2O_2 (or with the hydroxyl group of water) to produce Fe^{3+} and $^{\bullet}OH$ (Koppenol 2001; Lipinski 2011). Besides iron, other metals can participate in those reactions, such as copper and zinc (Das et al. 2015).

Haber-Weiss reaction: $Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$

Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

Net reaction: $O_2^{\bullet-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2$

Additionally, the hypoxic environment in the mitochondrial respiratory chain also can lead to the production of nitric oxide (NO), which can originate reactive species, such as RNS and reactive aldehydes that induce extreme lipid peroxidation (Reuter et al. 2010; Vera-Ramirez

et al. 2011). However, several exogenous sources of free radicals also contribute to oxidative stress development, as for example tobacco smoke, ultraviolet radiation, air pollutants, or ionizing radiation (Raham et al. 2012).

Typically, there is an equilibrium between the generation of free radicals and ROS and the antioxidant defence system, named oxidative balance. When a disturbance in the pro-oxidant/antioxidant balance in favour of the former occurs, oxidative stress may arise (Khan 2002; Goodman et al. 2007; Chang et al. 2008; Vera-Ramirez et al. 2011). Oxidative stress can lead to the damage of cellular macromolecules (*e.g.* DNA, proteins and lipids), contributing to cellular death, with serious impact in the whole organism (Fig. 1.2) (Limón-Pacheco and Gonsebatt 2009; Reuter et al. 2010). In fact, ROS and RNS can disrupt mitochondrial respiration and induce mitochondrial damage, which is thought to be related to neurodegeneration, namely in Alzheimer's and Parkinson's diseases (AD and PD, respectively). Moreover, oxidative stress can enhance the development of chronic inflammation, cancer and diabetes (Pietta 2000; Kohen and Nyska 2002; Singh et al. 2005).

To attenuate the oxidative stress-induced damage, aerobic organisms have developed enzymatic and non-enzymatic antioxidants defence systems. Enzymatic defences include catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), and repair enzymes (*e.g.* methionine sulfoxide reductase or 3 0-repair diesterase). Non-enzymatic systems comprise compounds with inherent antioxidant properties that reduce free radicals and ROS by electron donation, such as vitamins A, E and C, flavonoids, bilirubin and uric acid and other small molecules derived from natural sources, and that chelate pro-oxidant metal ions like iron and copper, such as specialized proteins (Lopaczynski and Zeisel 2001; Khan 2002; Arsova-Sarafinovska et al. 2009; Limón-Pacheco and Gonsebatt 2009). For this reason, one approach to prevent oxidative stress-related diseases (*e.g.* diabetes, neurodegeneration) is the use of antioxidants that stabilize or deactivate free radicals/ROS before cellular damages occur and, thus, are pivotal in maintaining the ideal cellular and systemic conditions. Also, chelation of redox metals can prevent the generation of ROS, protecting the organism from ROS production and reducing the occurrence of the above-mentioned diseases (Kohen and Nyska 2002). In this sense, studies on natural antioxidants as nutritional supplements and health foods have gained greater importance, due to their lower toxicity and side effects compared to synthetic ones. Antioxidants are probably the most useful compounds found in halophyte natural extracts. In fact, diverse halophyte species are reported with potent antioxidant activity, as for example *A. halimus*, *M. edule, E. maritimum, A. campestris subsp. maritima, Helichrysum italicum* subsp. *picardii* (Boiss. & Reut.) Franco*, P. coronopus* or *C. maritimum* (Custódio et al. 2012; Oueslati

et al. 2012; Amessis-Ouchemoukh et al. 2014; Jallali et al. 2014; Rodrigues et al. 2014; Stanković et al. 2015; Qasim et al. 2016; Pereira et al. 2017a,b,c, 2018, 2019; Petropoulos et al. 2018a,b; Zengin et al. 2018).

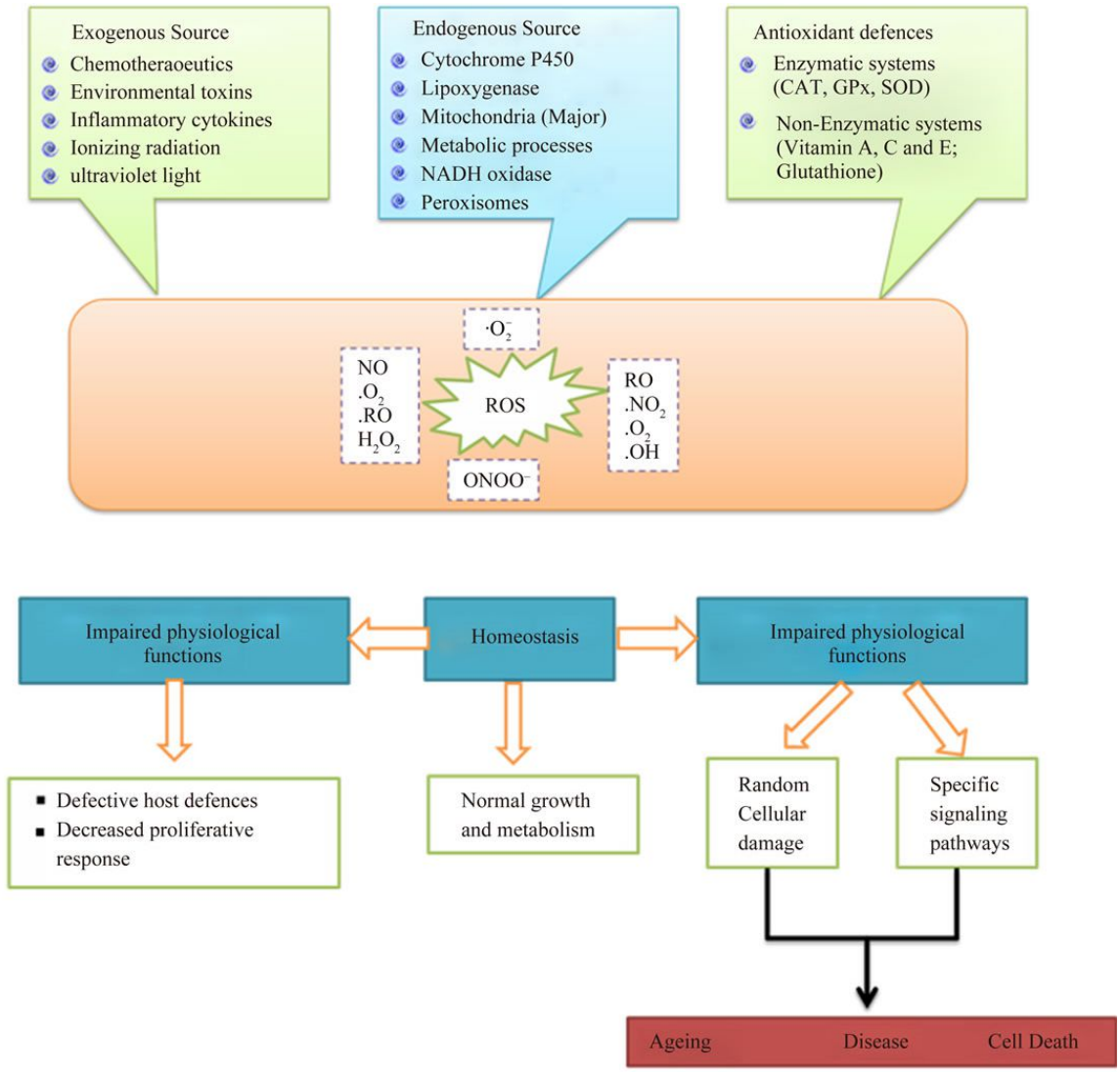

Figure 1.2. Source and cellular responses to reactive oxygen species (ROS) (Rahman et al. 2012).

1.5.2. Anti-inflammatory activity

Inflammation is a host's response against injury and/or infectious agents, such as viruses or bacteria, which involves the migration of blood cells into tissues. This response leads to hyperaemia (redness), increased permeability of microvasculature and leakage of protein into the interstitial space (swelling), increased blood flow and metabolic activity of cellular mediators (heat), and changes in the perivasculature and associated nerve endings (pain) (Fig.

1.3) (Libby 2007). Inflammation also comprises interactions between different types of cells, soluble mediators and tissue matrix. Platelets, basophils, fibroblasts, lymphocytes, as well as phagocytic, dendritic, mast, endothelial and smooth muscle cells are involved in the process (Kushner 1998). Signalling molecules also participate in inflammation, such as immunoglobulins, arachidonic acid metabolites, complement components, coagulation factors, fibrinolytic molecules, histamine and other vasoactive amines, lysosomal enzymes and neutrophil-derived products (defensins, cytokines, chemokines and their modulators, neuropeptides, reactive oxygen intermediates and NO) (Kushner 1998). Notably, macrophages are important phagocytic cells that rapidly respond to stimuli generated by injury or infection. Activated macrophages have enhanced microbiocidal capacity due to their ability to produce high levels of pro-inflammatory cytokines and mediators, which coupled with the natural killer (NK) cells' ability to produce increased amounts of superoxide anions and oxygen and nitrogen radicals, increases the macrophages phagocytic capacity (Mosser and Edwards 2008). For these reasons, the initial inflammation is often good in case of microbial infections, for example, but certain conditions can cause long-term inflammation, which is known as chronic inflammation (Fig. 1.3) (Hotamisligil 2006). Recently, researchers have found diverse inflammatory markers in multiple human diseases associated with ageing (*e.g.* cancer, diabetes, cardiovascular disorders, AD, rheumatoid arthritis, Crohn's disease and ulcerative colitis), comprising an important approach to understand the development of diseases and learn how to manage them (Fig. 1.3) (Blasko et al. 2004; Libby 2007; Holmes et al. 2009).

Complementary and alternative medicines often use natural products to prevent and treat diseases and preserve health. Several of these products have been employed over thousands of years, but only recently scientists started to understand their mechanism of action. One reason for the successful use of these medicines to prevent and treat chronic inflammatory diseases is the targeting of crucial players in inflammation (Hofseth 2008). In this context, *in vitro* cultured macrophages are frequently stimulated with lipopolysaccharide (LPS), a cell wall endotoxin produced by Gram-negative bacteria, becoming activated to produce inflammatory mediators, such as NO (Martich et al. 1993), a radical often associated with chronic inflammation (Kubes 2000; Joo et al. 2014). A decrease in NO production indicates the potential of natural products to reduce a chronic inflammatory response (Joo et al. 2014). On the other hand, an increase in NO production can imply an immunostimulatory effect of the extracts, enhancing the macrophage defence system to cope with a microbial infection, for example (Wink et al. 2011). With this purpose, there is a recent trend to find new molecules, food and beverages that could be used as dietary products to improve health by decreasing inflammation, and some may

include halophyte species, as for example, *Sarcocornia fructicosa*, *Cynodon dactylon* (L.) Pers., *Halimiones portulacoides* (L.) Aellen, *P. coronopus* or *T. gallica* that have been described with *in vitro* anti-inflammatory properties (Oueslati et al. 2012; Rodrigues et al., 2014; Petropoulos et al. 2018a).

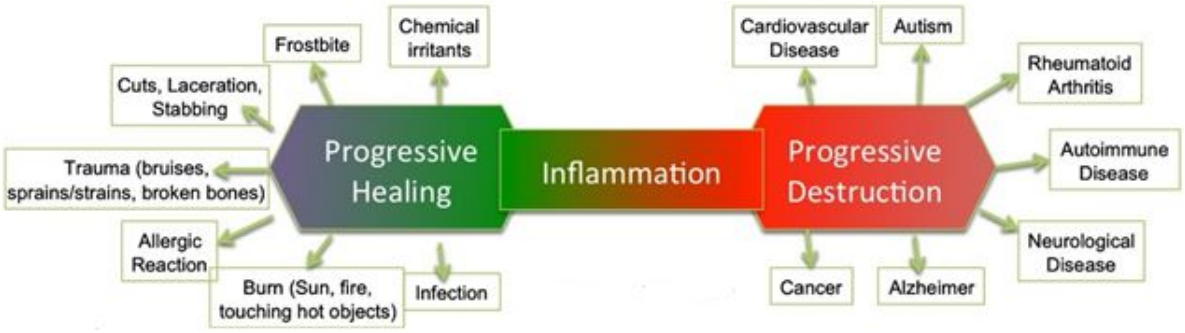

Figure 1.3. Inflammation in health and disease (Peterson 2012).

1.5.3. Neuroprotective potential

Neurodegeneration comprises a vast array of conditions affecting neuronal cells in the human brain. Neurons are crucial for nervous system functioning, but generally, they do not divide or are replaced, so when they are damaged or die, they cannot be substituted (Alzheimer's Association 2015). For this reason, neurodegenerative diseases are incurable and lead to progressive degeneration of the nervous system, which contributes to severe difficulties in daily living activities (Alzheimer's Association 2015).

AD occurs mainly in the elderly population (> 65 years of age) and is characterized by memory loss, thinking impairment, behaviour alterations and loss of the ability to perform daily living activities. AD is the most common type of dementia, comprising 60 to 80% of all cases and is officially considered the 6th leading cause of death in developed nations, after heart disease, cancer and stroke (Natarajan et al. 2009). In 2015, more than five million people were expected to have AD, including those under 65 years (Alzheimer's Association 2015). Although AD has no cure, its progression can be delayed and symptoms reduced, improving patients' quality of life (Alzheimer's Association 2015).

AD pathophysiology comprises the death of neurons, especially in the hippocampus region, related to memory and learning. Although the complete aetiology of AD is unknown, AD has two main hallmarks: deposits of amyloid-beta proteins and of Tau proteins (Fig. 1.4). The amyloid beta peptide is involved in brain development, but when it is highly concentrated,

its conformational changes contribute to the formation of amyloid fibrils that deposit outside neurons composing the senile plaques, which in turn leads to neurotransmission loss. Tau proteins, on the other hand, accumulate inside nerve cells in aggregates called neurofibrillary tangles, which are associated with extracellular amyloid plaques (Fig. 1.4) (Duthey 2013; Kumar et al. 2015).

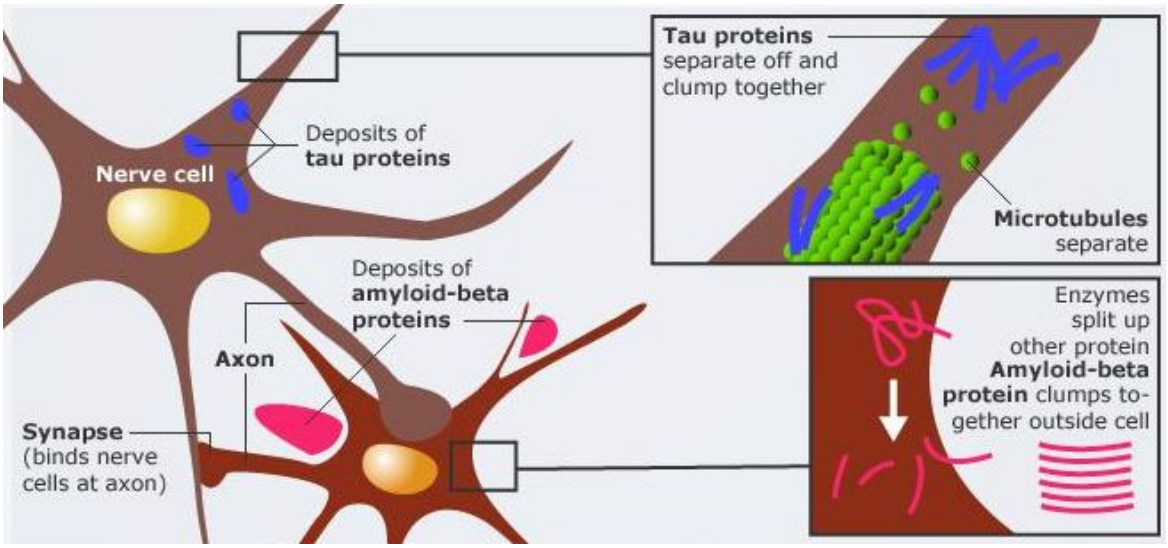

Figure 1.4. Main hallmarks of Alzheimer's disease (German Centre of Neurodegenerative disease 2019).

Biochemically, AD is characterized by a reduction in the levels of the neurotransmitter acetylcholine (ACh), that is responsible for decreased neurotransmission, which in turn causes insufficient transmission of the neurological impulse involved in muscle contraction, memory and learning capabilities (Houghton et al. 2006). The 'cholinergic hypothesis' states that ACh is hydrolysed, in the synaptic cleft, by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes into choline and acetate (Fig. 1.5) (Ballard 2002; Greig et al. 2005). The inhibition of these enzymes results in increased ACh levels in the synaptic cleft, contributing to improve the cognitive function and to decelerate disease progression (Tariot et al. 2000; Ballard 2002; Greig et al. 2005; Huang and Mucke 2012; Kumar et al. 2015). Cholinesterase inhibitors can act selectively against AChE (*e.g.* donepezil), BuChE (*e.g.* cymserine analogues), or can be dual inhibitors (AChE and BuChE, *e.g.* rivastigmine) (Fig. 1.5) (Ballard 2002; Greig et al. 2005). However, these types of drugs have a vast array of side-effects (*e.g.* hepatotoxicity, gastrointestinal, cardiorespiratory, extrapyramidal, genitourinary, and musculoskeletal), which drives the need for novel and less toxic treatments. Novel specific BuChE inhibitors and/or dual

enzyme inhibitors are being recently investigated as an effective and promising way to manage AD, aiming to maintain or improve the cholinergic activity in the brain, contributing to the amelioration of AD patients' life quality (Ballard 2002; Greig et al. 2005; Lane et al. 2006; Anand and Singh 2013; Geldenhuys and Darvesh 2015). In fact, several currently approved drugs for AD management were derived from natural products, such as physostigmine (Exelon®) and galanthamine (Nivalin®), naturally produced by *P. venenosum* and *G. caucasicus,* respectively. Regarding halophytes, several species already demonstrated potential *in vitro* neuroprotective properties, as for example *E. maritimum*, *P. lentiscus, Thespesia populnea* (L.) Sol. ex Corrêa, *A. macrostachyum* and *M. edule* (Amessis-Ouchemoukh et al. 2014; Rocha et al. 2017).

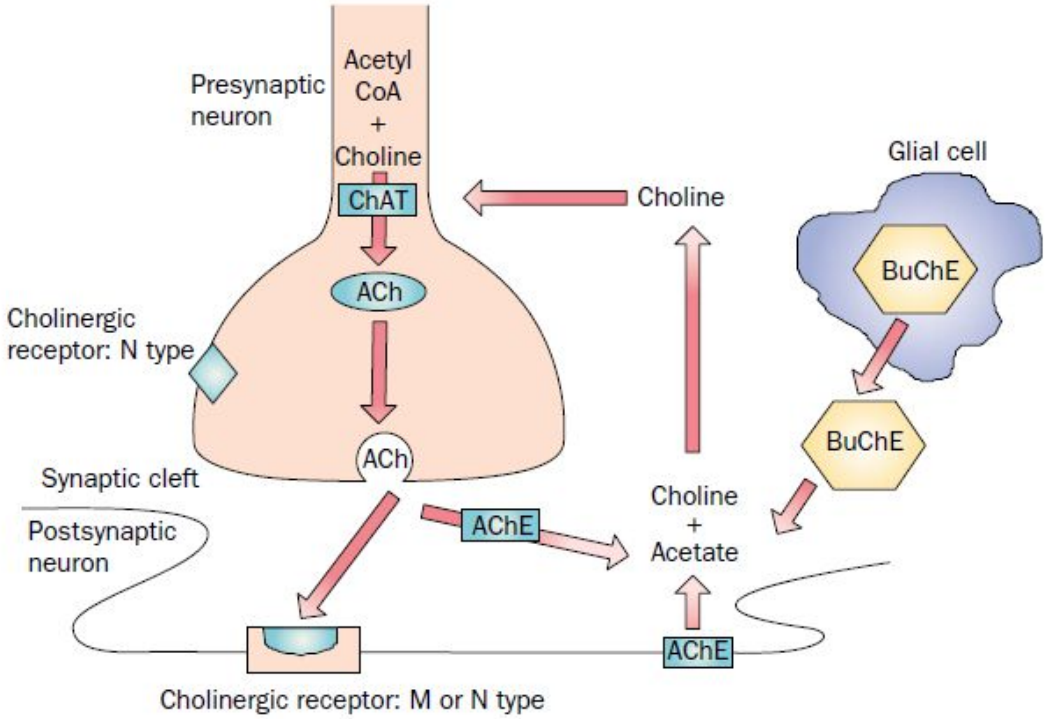

Figure 1.5. Scheme of the "cholinergic hypothesis" (Scarpini et al. 2003).

1.5.3.1. Oxidative stress and inflammation in neurodegeneration

There is evidence of the link between oxidative stress and the development and progression of different forms of dementia, such as AD and PD. The human brain is characterised by a high lipid content and oxygen metabolism, and by reduced levels of antioxidant defences. ROS are constantly formed in that organ by excitatory amino acids and neurotransmitters and result in oxidative stress and in injuries to neuronal and glial cells (Uttara et al. 2009). Moreover, transition metals, such as iron and copper, can accept or donate

electrons, which induces ROS formation, contributing to oxidative stress states and to disease progression (Chen et al. 2012). In this sense, compounds with antioxidant properties, as well as metal chelation capacity, are considered effective in the prevention of brain oxidative damage and neuronal loss associated with neurodegeneration (Tabner et al. 2005; Uttara et al. 2009). AD and PD pathogenesis are also associated with neuroinflammation, occurring as an inflammatory response from the central nervous system to brain damage, promoting the accumulation of activated astrocytes and microglial cells that produce pro-inflammatory mediators (Morales et al. 2014; Pasqualetti et al. 2015). Actually, some studies have demonstrated that the administration of anti-inflammatory drugs is related to a decreased risk of neurodegeneration (Morales et al. 2014).

1.5.4. Antidiabetic properties

Diabetes mellitus affects more than 350 million people worldwide, corresponding to approximately 9% of the world population, and is considered an emerging health issue in western societies. It is expected, by 2030, to be the 7th cause of death in the world (Mathers and Loncar 2006; Mai and Chuen et al. 2007; Danaei et al. 2011). Around 90% of all diabetes cases are diagnosed as Type 2 *diabetes mellitus* (T2DM), which is often linked with genetics and lifestyle features (Mozaffarian et al. 2009). The major aspect of T2DM pathophysiology is the high blood glucose level, called hyperglycaemia, which results from congenital or acquired deficiencies in insulin secretion, or from reduced responsiveness of the organs to the secreted insulin (WHO 1999; Yarchoan and Arnold 2014). For this reason, insulin injections are often used to manage T2DM, but there are other medications suitable for the control of the disease (American Diabetes Association 2015), such as inhibitors of carbohydrate-hydrolysing enzymes (*e.g.* α-amylase and α-glucosidase) that decrease the blood glucose levels by inhibiting the breaking down of starch into sugar monomers (Fig. 1.6) (American Diabetes Association 2015). These inhibitors contribute to the management of hyperglycaemia linked to T2DM by decreasing the postprandial increased blood glucose levels (Kwon et al. 2007). However, clinically used α-amylase and α-glucosidase inhibitors, as for example acarbose, miglitol and voglibose, cause several adverse side-effects, including abdominal distension, flatulence and meteorism (Bischoff et al. 1985). In this sense, it is essential to identify innovative and effective compounds with antidiabetic properties and reduced side-effects, as for example, from natural sources (Kwon et al. 2007). In this context, several halophyte species were recently identified as promising sources of carbohydrate-hydrolysing enzyme inhibitors, including *A.*

macrostachyum, E. maritimum, A. campestris subsp. *maritima, H. picardii, Halimione portulacoides* (L.) Aellen, *Salicornia europaea* L., *Suaeda fruticosa* Forssk. ex J.F.Gmel. and *Capparis decidua* (Forssk.) Edgew (Pereira et al. 2017a,b, 2019; Zengin et al. 2018).

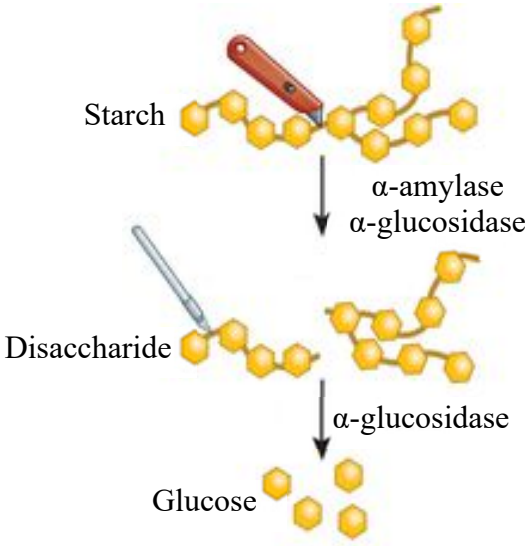

Figure 1.6. Scheme of the starch digestion process (adapted from Bernardi and Sattin 2015).

1.5.4.1 Oxidative stress and inflammation in diabetes

Hyperglycaemia, which is typical of T2DM, induces diverse metabolic alterations that lead to the development of oxidative stress and chronic inflammation, contributing to the development of diabetes-associated complications, such as cardiovascular, urological, neurological, renal and ophthalmological disorders (Mai and Chuyen 2007; American Diabetes Association 2010; Vikram et al. 2014). When the antioxidant defence mechanisms of the organism are reduced, oxidative stress can arise and enhance the damage caused by free radicals and ROS, favouring the disease progression (Sabu and Kuttan 2002; Maritim et al. 2003). Moreover, ROS and free radicals also provide an appropriate environment to stimulate the production of pro-inflammatory mediators (*e.g.* cytokines and chemokines), which contribute to induce insulin resistance (Akash et al. 2013; Muriach et al. 2014). In this context, the identification and development of novel and combined therapies aiming at the prevention of oxidative and inflammatory states are indicated as a novel and promising strategy to manage T2DM (Ruhe and McDonald 2001; Devasagayam et al. 2004; Fardoun 2007; Akash et al. 2013).

1.5.5. Anti-hyperpigmentation properties

Melanin is produced and distributed in the skin and hair follicles and its main role is protection against ultraviolet (UV) radiation. However, its overproduction and accumulation can lead to the development of high pigmented patches, namely melasma, freckles, post-inflammatory melanoderma and solar lentigo (Hakozaki et al. 2015; Khan 2007; Ribeiro et al. 2015; Tu and Tawata, 2015). Melanin biosynthesis is modulated by the multifunctional copper-containing enzyme tyrosinase, which is an important regulator and rate-limiting enzyme of melanogenesis (Fig. 1.7) (Khan 2007; Hakozaki et al. 2015; Ribeiro et al. 2015; Tu and Tawata 2015). In this sense, products and/or compounds able to reduce melanin synthesis, such as tyrosinase inhibitors, have extensive cosmetic applications as skin-whitening products to treat excessive melanin accumulation (Hakozaki et al. 2015). Besides, tyrosinase is also responsible for the disagreeable browning of fruits and vegetables in post-harvest processing, which decreases their market value (Khan 2007; Chang 2009), and therefore, compounds with tyrosinase inhibitory capacity are also of interest for the food industry.

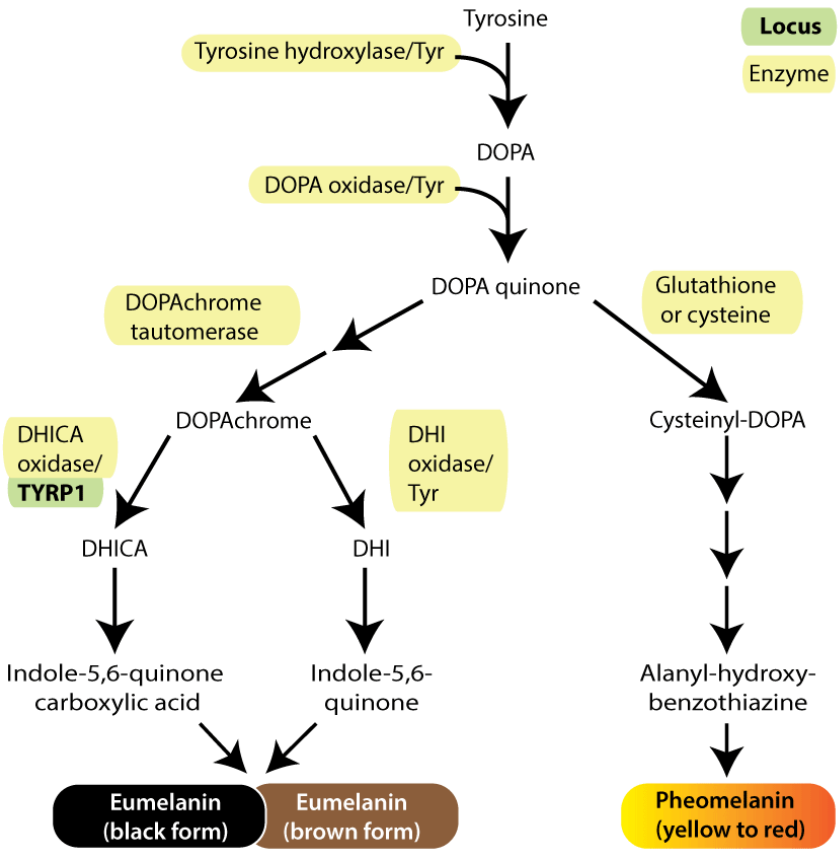

Figure 1.7. Scheme of melanin synthetic pathway (Watson et al. 2015).

The cosmetic market is a highly innovative sector focused on science-driven novel products development, which allows the creation or redesign of excellent and innovative ingredients corresponding to the ever-changing consumers' expectations. For instance, every year a quarter of all marketed cosmetic products are improved or are completely new. To correspond to this ever-increasing demand, research has been recurring to traditional natural ingredients to develop novel formulations, contributing to reduce the possible side-effects of the products and increase their acceptance by the consumers (Cosmetics Europe 2017). Thus, natural products with skincare applications, as anti-ageing, anti-wrinkles or skin-whitening for example, are on high demand. Some natural-derived molecules are commercially available to use as tyrosinase inhibitors, such as kojic acid, arbutin and hydroquinones (Khan 2007; Chang 2009; Hakozaki et al. 2015; Ribeiro et al. 2015). Several halophyte species were previously identified as potential sources of compounds for skin care, with industrial cosmetic applications, displaying relevant biological activities such as *in vitro* antioxidant, anti-inflammatory and antimicrobial (*e.g. P. lentiscus*, *C. maritimum*, *Lythrum salicaria* L., *Limonium wrightii* (Hance) Kuntze., *M. edule*, *Limoniastrum monopetalum* (L.) Boiss., *T. gallica* and *S. ramosissima*) (Ksouri et al. 2012; Guillerme et al. 2017). Specifically, the species *P. lentiscus, A. campestris* subsp. *maritima* and *A. pungens* exhibited a high capacity to inhibit tyrosinase, with interest to prevent or manage undesired skin and food darkening (Lopes et al. 2016; Rodrigues et al. 2018; Pereira et al. 2018).

1.5.6. Antitumor properties

Cancer is one of the most deadly diseases in developed and developing countries, with 60 million deaths occurring every year worldwide, and it is expected to increase in 70% in the next two decades (WHO 2015). It is a major public health problem because most cancers are incurable at the time of diagnosis (Khan 2002). However, at least one-third of cancers (> 7 million) can be prevented, since they are caused by lifestyle features such as tobacco and alcohol consumption, physical inactivity, dietary factors, obesity, infections, and exposure to environmental pollution, occupational carcinogens and radiation (WHO 2015).

Cancer is a disease of the body's own cells. Although cellular proliferation is tightly controlled, cellular components can be damaged in such a way that cells are able to escape from checkpoints regulating cell division and adhesion. Uncontrolled multiplication of these damaged cells, which might contain mutations in their genome, can lead to tumour formation. Moreover, cancer is not a single disease but rather a panoply of several diseases sharing an

essential property, which is deficient control of cellular growth (Kenny 2007). Abnormal growth of cells, caused by multiple changes in gene expression, leads to a deregulated balance of cell proliferation and cell death, and finally evolves into a population of cells with the potential to invade tissues and metastasize to distant sites (Ruddon 2007; Ullah and Aatif 2009).

1.5.6.1. Hepatocellular carcinoma (HCC)

HCC is the third leading cause of cancer deaths worldwide, and around half a million people are affected with this type of cancer every year. HCC is considered a primary malignancy of the liver and mainly occurs in people suffering from chronic liver diseases, such as cirrhosis or hepatitis (Gomes et al. 2013). It is one of the most aggressive types of cancer characterised by a high metastatic potential, as well as resistance to cytotoxic agents. If the tumour has not spread, it can be removed by surgery, but only 10 to 20% of HCC can be completely removed; if not, the life expectancy rarely exceeds 6 months (Gomes et al. 2013). Despite the recent advances, sorafenib is the only molecular inhibitor approved by FDA to treat the advanced HCC, but other chemotherapeutics were routinely used alone or in combination, namely doxorubicin, epirubicin, cisplatin, 5-fluorouracil and etoposide; however, they all show very low efficacy. For example, sorafenib only increases the life expectancy by 3 months (Cao et al. 2012). These reasons drive the need for developing new leading compounds for HCC chemotherapeutics. The chemical diversity and structural complexity of natural products make them ideal candidates for the identification of novel chemotherapeutics. Many phytochemicals have been described with interesting anti-tumour properties and provided multiple active compounds in the past; several drugs are in clinical use for cancer treatment like paclitaxel (Taxol®) and vinblastine (Velban®) (Deorukhkar et al. 2007). Recently, the marine environment also became a promising source of novel biologically active molecules, including microorganisms, algae, invertebrates or halophytes (Blunt et al. 2013). Some halophytes were already reported to display *in vitro* antitumor activity against human cell lines, highlighting their potential in this field, as for example the species *Limonium densiflorum* (Guss.) Kuntze, *T. gallica, S. fruticosa* and *Salicornia herbacea* L. (Oueslati et al. 2012). The phenanthrene 1,7-dimethyl-5-vinyl-9,10-dihydrophenanthren-2-ol, shortly called juncunol, was isolated from the aerial part of the halophyte *J. acutus* with high cytotoxicity against hepatocellular carcinoma cells and with high selectivity, i.e., with low toxicity towards non-tumoral cells (Rodrigues et al. 2014).

1.5.6.2. Apoptotic mechanisms

Failure of apoptosis is a main feature of the carcinogenic process, thus, one strategy underlying anticancer drug development is the induction of the apoptotic machinery in cancer cells. Apoptotic cell death is induced or pre-programmed, causing the death of individual cells (Zimmermann et al. 2001). These apoptotic cells display distinctive morphological features, namely blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding, cell shrinkage, and phosphatidylserine exposure on the outer leaflet of the plasma membrane, which allow their recognition and removal by phagocytic cells (Fig. 1.8) (Wu et al. 2004; Lee et al. 2013).

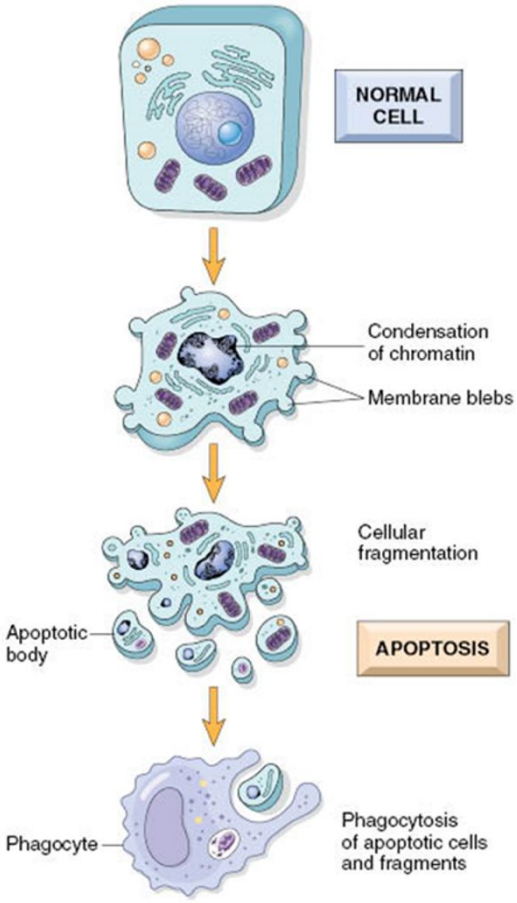

Figure 1.8. Apoptotic morphological features (Kumar et al. 2010).

The decrease in the mitochondrial membrane potential due to oxidation-reduction changes has also been linked to apoptotic cell death in several cell types, including liver cells (Fulda et al. 2010). The apoptotic pathway is associated with alterations in the mitochondrial

membrane potential (Δψm), which leads to mitochondrial membrane permeabilization, the release of cytochrome c and caspase activation (Waterhouse et al. 2001). Likewise, the correct cell cycle progression is essential for cell division and proliferation and is regulated by checkpoints that ensure the detection of cell damage that can be repaired, or when it is not possible, it activates pathways that induce the programmed cellular death. Alterations in the cell cycle checkpoints usually lead to mutations contributing to cancer development. Thus, the use of chemotherapeutic agents usually contributes to the cell cycle arrest, decreasing the tumour cells' growth and proliferation and inducing apoptosis (Pietenpol and Stewart 2002).

1.5.7. Toxicological evaluation

Natural products, as for example herbal ingredients, are usually considered safer than synthetic ones and linked to reduced side effects (Karimi et al. 2015). However, a toxicological evaluation is of utmost importance to assure their safety, and usually, *in vitro* methods targeting the assessment of cytotoxicity towards tumoral and non-tumoral mammalian cell lines are recommended (Nogueira et al. 2011; Liu et al. 2018). These methods include, for example, determining cell viability through the evaluation of cellular metabolic activity and plasma membrane integrity when exposed to the botanical or a purified compound (Nogueira et al. 2011; Liu et al. 2018). One of the most simple, quick, consistent, sensitive, and cost-effective approach for preliminary toxicity screenings is the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This is a colorimetric method that evaluates the cell metabolic state, giving information about the potential toxicity of a certain sample (Liu et al. 1997; Saad et al. 2006; Nogueira et al. 2011). In fact, some authors have established a positive correlation between *in vitro* toxicity towards mammalian cell lines and *in vivo* toxicity determined in animal models, which allows reducing animal testing (Carballo et al. 2002; Parra et al. 2001). Additionally, the toxicological evaluation of natural compounds can be complemented using the brine shrimp *Artemia salina* L. as toxicity *in vivo* model (Parra et al. 2001; Carballo et al. 2002). Therefore, it is accepted that if a botanical sample or compound have reduced or no cytotoxicity on these models, they can be considered safe for consumers (Parra et al. 2001; Carballo et al. 2002). In addition, if plants are reported with uses in traditional medicine or as food, it may be an indication that they have low toxicity and their consumption is safe.

1.6. Plant bioactive compounds

Plant bioactive compounds are generally produced as secondary metabolites, which are not required for plant growth and development but have important functions in plants, for example, protecting against oxidative damage, promoting pollination, inhibiting competing plants, or avoiding herbivory (Hutzler et al. 1998). These substances may belong to different categories of chemical groups, namely glycosides, terpenoids, alkaloids, phytosterols and phenolic compounds (Ksouri et al. 2012). In addition to their physiological roles, secondary metabolites also display interesting pharmacological or toxicological activities, such as antioxidant, antidiabetic, neuroprotective, antitumor or anti-inflammatory (Ksouri et al. 2012). However, primary metabolites (carbohydrates, amino acids, proteins and lipids) that are used for normal plant growth and maintenance can also display biological properties relevant for human health (Bernhoft 2010).

1.6.1. Polysaccharides

Polysaccharides are long-chain carbohydrates constituted by monosaccharides connected through glycosidic bonds. Usually, one polysaccharide contains among 200 - 2500 monosaccharides, grouped in a linear or branched carbon chains, and can be classified according to their chemical structure as homo-polysaccharides, constituted by a single monosaccharide, or as hetero-polysaccharides, formed by different monosaccharide units (Liu et al. 2015). Moreover, they are usually associated with other biomolecules, including proteins, polynucleotides, lipids, lignin, or inorganic minerals (Liu et al. 2015).

In plants, polysaccharides can have structural functions, as for example cellulose that is part of plant cell walls, or have energy storage purposes, such as starch, a glucose polymer formed by amylose and amylopectin (Liu et al. 2015). Furthermore, diverse types of polysaccharides are also used in food, feed and pharmaceutical industries, as for example xylans or xyloglucans for drug delivery applications, or pectin as gelling or thickening agents for the food industry or as an excipient for the pharmaceutical industry (Liu et al. 2015). In halophytes, some polysaccharides have been identified with beneficial health effects, such as antioxidant and anti-inflammatory actions. For example, an *S. herbacea* polysaccharide-rich extract was reported to have *in vitro* immunostimulatory properties by the stimulation of macrophages to produce NO, inducible NO synthase (iNOS) through NF-κB activation (Im et al. 2006). Moreover, a pectin-like polysaccharide (galactoarabinan) isolated from *S. fruticosa* is described

with *in vitro* antioxidant, and *in vivo* anti-inflammatory and antinociceptive properties (Mzoughi et al. 2018).

1.6.2. Glycosides

Glycosides are molecules with a sugar unit linked to a functional group by a glycosidic bond. They have different roles in plants, including for example storage of different substances in the form of alcohol, anthraquinone, coumarin, chromone, cyanide, or phenolic glycosides (Bartnik and Facey 2017). Diverse plant glycosides, particularly from halophyte species, have demonstrated biological properties important to human health, such as cancer prevention, antidiabetic and antibacterial effects (Ksouri et al. 2012). For example, glucosinolates (glucotropaeolin, 2-methyl butyl glucosinolate, ethyl glucosinolate and 4-pentyl glucosinolate) isolated from *C. maritima* exhibited DPPH antiradical, molluscicidal and antifungal activities (Sellam et al. 2007; Radwan et al. 2008). Standardised preparations of *Urginea maritima* (L.) Stearn, used in the treatment of cardiac and renal insufficiencies, contains glucoscillarene A, proscillaridine A, scillarene A, scilliglucoside and scilliphaeoside, as well as flavonoids and polysaccharides as the bioactive compounds (Adams et al. 2009). Saponins (amphipathic glycosides) also have pharmacological properties, including haemolytic, antibacterial, immunological and antidiabetic (Ksouri et al., 2012). For example, four triterpenoid saponins (including 3β-hydroxy-23-oxo-30-noroleana-12, 20(29)-diene-28-oic acid 3-O-β-D-glucuronopyranosyl-28-O-β-d-glucopyranoside) were isolated from the n-butanolic fraction from the aerial parts of *S. herbacea,* displaying *in vitro* antioxidant properties (Kim et al. 2012).

1.6.3. Lipids

Lipids are hydrophobic or amphiphilic small molecules and include diverse types of compounds, such as waxes, sterols, fat-soluble vitamins, mono, diglycerides and triglycerides, phospholipids, carotenoids and fatty acids (saturated, monounsaturated or polyunsaturated). Their main biological functions are energy storage, cell signalling, as cell membrane structural constituents, and as precursors of steroid hormones. Besides, they also have health beneficial effects and have numerous applications in food (*e.g.* emulsifiers or health supplements) and cosmetic industries (*e.g.* moisturizing or as excipients) (Alvarez and Rodríguez 2000).

Halophyte seeds are particularly rich sources of fat and vitamins, particularly of unsaturated fatty acids (FAs), including some polyunsaturated FAs, such as α-linolenic, linoleic

and oleic acids. Indeed, the seeds of the halophyte species *C. maritimum* are rich in PUFAs, particularly in oleic acid that constitutes 81% of all fatty acids (Zarrouk et al. 1996) and is known to have antitumor, hypoglycaemic and anti-inflammatory properties (Vassiliou et al. 2009; Carrillo et al. 2012; Su et al. 2013). *Zygophyllum album* (L.f.) Beier & Thulin seed oils, on the other hand, are mainly constituted by linolenic acid (64%) (Zarrouk et al. 2003), that was described with *in vivo* antioxidant, and *in vitro* anti-inflammatory and antidiabetic activities (Ren and Chung 2007; Richard et al. 2008; Su et al. 2013). Moreover, the long chain monoenoic FAs demonstrated to be effective in the treatment of demyelinating diseases, since they promote the myelin biosynthesis (Sargent et al. 1994).

Phytosterols are minor compounds present in plant oils, but they have diverse beneficial effects. For example, β-sitosterol has a cholesterol-lowering activity, contributing to decreasing the risk to develop cardiovascular problems (Patel and Thompsom 2006). Diverse authors reported the isolation of phytosterols from halophytes, particularly from different *Teucrium* species, where clerosterol, poriferasterol, campesterol, stigmasterol and sitosterol were identified (Gaspar et al. 1996; Hachicha et al. 2009). These compounds have been described with the capacity to reduce the LDL-cholesterol levels by competing for its intestinal absorption, which agrees with the folk medicine records about the hypolipidemic effect of *Teucrium* species (Ntanios et al. 2003).

1.6.4. Alkaloids

Alkaloids are molecules containing nitrogen in heterocyclic rings and are the largest class of plant secondary metabolites. They display important biological activities, such as antitumor, antimicrobial or anti-parasitic, and several were the basis for the synthesis of some of the most known and used drugs worldwide for the treatment of different diseases (Aniszewski 2007). For example, morphine and codeine were first isolated from *Papaver somniferum* L. and are now chemically synthesized and used as an analgesic (morphine) or as antitussive (codeine). Galantamine was firstly isolated from the plant *G. caucasicus* and is currently used for AD treatment (Ng et al. 2015), whereas others were used for the treatment of cancer, namely paclitaxel (isolated from *T. brevifolia*) and vinblastine (derived from *C. roseus*) (Amirkia and Heinrich 2014).

Halophytes are also endowed with several bioactive alkaloids, for example, *Nitraria* species contain 13-earbolines, quinolizidines, spiropiperidines and quinazolines with *in vitro* antioxidant and hypolipidemic properties (Wang et al. 2007; Ksouri et al. 2012). Isochinolin

alkaloids (salsolin and salsolidin) were clinically used for the treatment of hypertonia and insomnia and were primarily identified in *Salsola kali* L. (Sokolowska-krzaczek et al. 2009). An alkaloid-rich fraction from *C. decidua* also has shown antidiabetic activity and reduced development of diabetic complications in animal tests (Sharma et al. 2010).

1.6.5. Phenolics

Phenolic compounds are secondary metabolites derived from the pentose phosphate, shikimate and phenylpropanoid pathways in plants (Balasundram et al. 2006). Their chemical structures comprise one or several condensed aromatic rings, bearing one or more hydroxyl groups, which can differ from simple phenols to highly polymerized molecules, usually called polyphenols (Fig. 1.9) (Dai and Mumper 2010). Its high structural diversity results in a wide range of compounds that can be classified in several categories, namely simple phenols, benzoquinones, hydroxybenzoic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, phenylpropanoids, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids, isoflavonoids, lignans, neolignanes, biflavonoids, lignins, and condensed tannins (Balasundram et al. 2006; Pandey and Rizvi 2009; Soto et al. 2015).

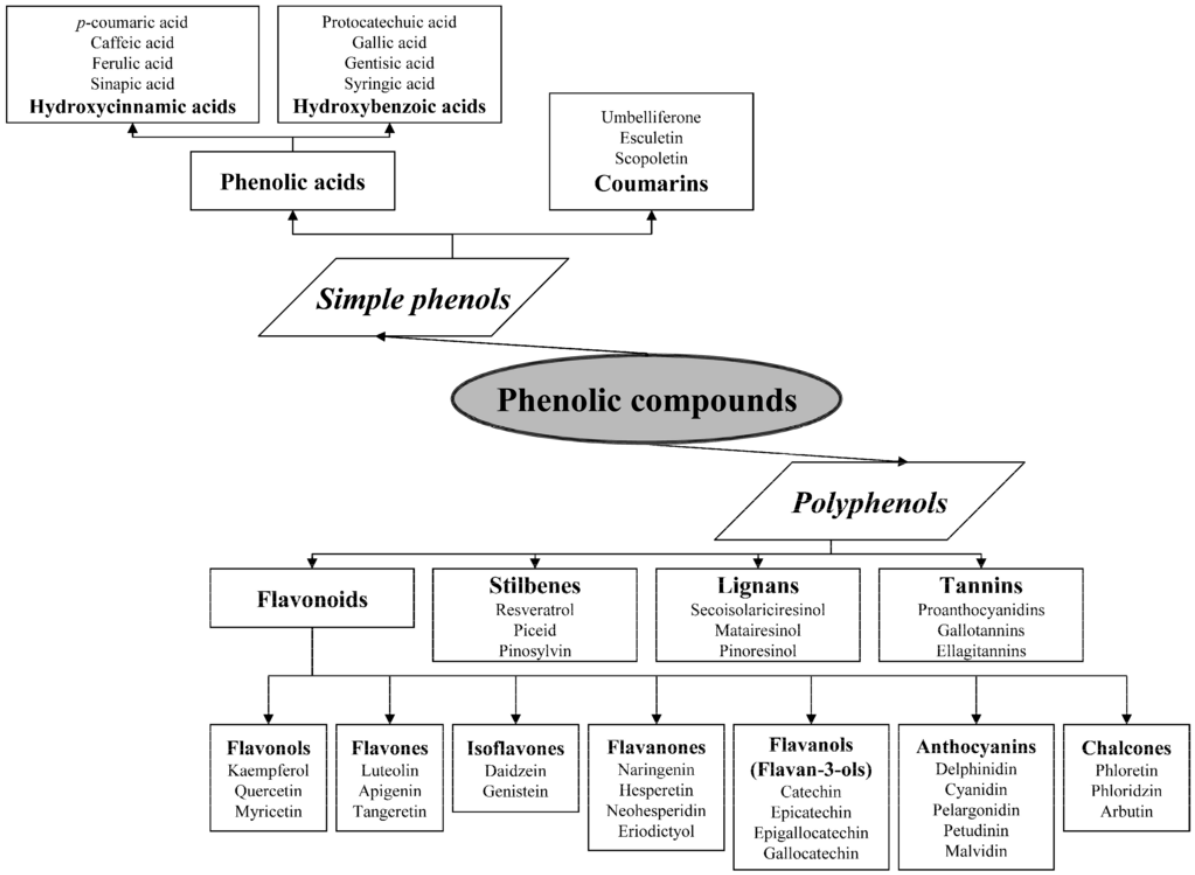

Figure 1.9. Different classes of plant phenolic compounds (Soto et al. 2015).

1.6.5.1. Physiological role

Phenolic compounds are the most widely distributed phytochemicals in the plant kingdom and participate in important physiological roles in plants (Balasundram et al. 2006). Phenolics are involved in many interactions between plants and the environment, for example against herbivory, and are accumulated 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, which contribute to pigmentation, attracting pollinators to increase fertilization rates, and animals to eat the fruits and spread the seeds (Hutzler et al. 1998; Mol et al. 1998; Pichersky and Gang 2000; Atmani et al. 2009). In the roots, flavonoids are usually involved in the defence mechanism against pathogens, which is probably related to their antimicrobial and antioxidant properties (Hassan and Mathesius 2012). In fact, flavonoid biosynthesis seems to be increased during fungal infections (Weston and Mathesius 2013). Flavonoids are usually constituents of roots exudates and may also act as a chemoattractant of symbiotic nitrogen-fixing bacteria or rhizobia, inhibiting or stimulating their gene expression and leading to nitrogen fixation in a convenient form to plants and a carbon source to the bacteria (Weston and Mathesius 2013). Moreover, root exudates containing flavonoids may stimulate spore germination, hyphal branching in the soil, and root colonization (Lattanzio et al. 2006; Weston and Mathesius 2013). They also act as metal chelators of soil components, contributing to increasing the phosphorus and iron availability to the plant (Hassan and Mathesius 2012; Weston and Mathesius 2013). Flavonoids also inhibit the growth and germination of other plant species, which is called allelopathy (Hassan and Mathesius 2012). In turn, in the leaves, flavonoids may confer protection against extreme UV radiation exposure, due to their UV-absorbing properties, confirmed by *in vitro* studies showing that UV exposure induced biosynthesis of flavonols, especially with higher hydroxylation levels in *Arabidopsis* plants (Winkel-Shirley 2002; Lattanzio et al. 2006).

Tannins can be present in diverse plant tissues, including twigs, wood, bark, roots, seeds and fruits. They are players in plant-insect interactions, through the decreasing of protein digestion that leads to defective larval performance, for example. Insect damage stimulates the biosynthesis of tannins in some plants, indicating their role in herbivory defence (Barbehenn and Constabel 2011). Additionally, tannins confer the roots with resistance to microbial decomposition due to their inhibitory effect on soil microbial activity (Kimura and Wada 1989); they also mitigate the excess iron-injury in that organ by forming a tannin-ferric iron complex that promotes the oxidation of hydrogen sulphide, reducing its toxicity to the roots (Kimura and

Wada 1989; Barbehenn and Constabel 2011). In the leaves, they can function as UV-screening compounds or antioxidants, as described for flavonoids (Barbehenn and Constabel 2011).

Hydroxycinnamic acids are usually present in the cell wall and there is some evidence that they are a reservoir for lignin biosynthesis, necessary to plant growth (Lattanzio et al. 2006). Besides, they also play a role as a mechanism of light signal transmission by interaction with control proteins, triggering alterations in the cell wall structure, water flux, turgor pressure and growth, and are known to have flowering-inducing properties (Lattanzio et al. 2006). Hydroxybenzoic acids also display allelopathic effects, avoiding the growth of other plant species like, for example, by inhibition of the seed germination through the decreasing of glycolytic enzymes activity and cellular respiration (Seal et al. 2004; Weir et al. 2004).

The wide range of physiological functions of phenolics, widely distributed in plants, makes them potentially biologically active compounds, providing important properties to plants, such as antioxidant, anti-inflammatory or antimicrobial (Balasundram et al. 2006).

1.6.5.2. Biological properties

Phenolic compounds are widely spread in plant foods (*e.g.* fruits, vegetables, cereals, olive oil, chocolate) and beverages (*e.g.* coffee, tea, herbal teas, beer, wine, juices), conferring organoleptic (*e.g.* colour, taste and smell) and biological properties (*e.g.* anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory) to these products (Balasundram et al. 2006; Dai and Mumper 2010). Their health benefits have become an increased focus of interest and are mainly associated with their strong redox properties and wide distribution in these plant ingredients (Dai and Mumper 2010). Countless *in vitro* studies have discussed their properties as reducing agents, hydrogen donors, singlet oxygen quenchers, and as metal chelators (Charles 2013). For these reasons, several studies has established an association between the consumption of polyphenolic-rich foods and/or beverages with a reduced risk of developing oxidative stress-related diseases, such as cancer, neurodegenerative diseases, T2DM, cardiovascular disorders, hypertension, obesity, osteoporosis, hepatic, renal and gastrointestinal diseases (Pandey and Rivzi 2009; Dai and Mumper 2010; Yordi et al. 2012).

However, some studies have reported that some phenolic compounds, in particular flavonoids and phenolic acids (*e.g.* quercetin, myricetin, kaempferol and caffeic, chlorogenic and ferulic acids), can have a dual behaviour, acting as antioxidants or pro-oxidants, depending

of some specific conditions namely high concentrations of certain phenolics or presence of metal ions (Yordi et al. 2012). For example, the presence of oxygen with iron and copper promotes redox cycling (repeated coupled reduction and oxidation reactions) of flavonoids, leading to ROS formation and consequent damage of cellular macromolecules (*e.g.* DNA, lipids and proteins) (Carocho and Ferreira 2013). Even so, recent studies indicated that their pro-oxidant action may be beneficial for specific cellular targets, like cancer cells, inducing mitochondrial dysfunction that leads to apoptosis (Yordi et al. 2012). However, the *in vivo* mechanisms of antioxidant/pro-oxidant effects of phenolics is still controversial, since there is a lack of clinical evidence of its efficacy, and their *in vivo* metabolism during digestion is not completely known (Sorriento et al. 2018).

Plants have proven to be excellent sources of phenolic antioxidants with similar activities to that of synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) (Balasundram et al. 2006). These compounds are often used to preserve foods and beverages, but there are several concerns regarding their safety (Pokorný 1991; McCarthy et al. 2001). For example, BHA was proved to induce lesions in the forestomach of rats, whereas BHT has shown to induce internal and external haemorrhages when ingested in high doses, causing the death of mice and guinea pigs (Shahidi and Wanasundara 1992). For these reasons, natural phenolic antioxidants, namely those with a single aromatic ring and flavonoid-derived molecules, can be used as substitutes of these synthetic preservatives, in the food and cosmetic industries (Galal 2006; Addor 2017; Roselló-Soto et al. 2018). Besides, phenolic compounds also display strong antimicrobial properties, preventing simultaneously chemical and microbiological deteriorations of foods, beverages and cosmetics, as for example hydroxycinnamic acids and flavonols. Even so, their use as food and cosmetic preservatives is not widely spread yet (Maqsood et al. 2014; Soto-Chilaca et al. 2016).

The biological activity of phenolic compounds, namely as antioxidants, is mainly dependent on the number and positions or their hydroxyl groups, as well as the type of substitutions on the aromatic rings. Diverse phenolic compounds were already reported in different halophyte species, from different families, displaying important biological properties: largely antioxidant, but also hypoglycaemic, antitumor, anti-inflammatory, antithrombotic, anti-ageing, cardioprotective, and antimicrobial (Ksouri et al. 2012).

Atriplex halimus was described with strong antioxidant activity, attributed to the presence of flavanols aglycones, such as kaempferol, quercetin, isorhamnetin, patuletin, spinacetin and tricin (Sanderson et al. 1988; Benhammou et al. 2009). Other phenolic

compounds, including rutin, hyperoside and ferulic acid were isolated from *Carpobrotus edulis* (syn. *M. edule*) shoots and have been described with strong antiradical activities against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Okuda 2005). *Artemisia scopariae* Waldst. & Kit. is mainly constituted by *p*-hydroxyacetophenone, chlorogenic and caffeic acids (Yao and Chen 2007), contributing to the antioxidant, anti-inflammatory, antitumor and antibacterial properties of this species (Ksouri et al. 2012). These compounds also explain its Chinese traditional use in the treatment of hepatitis, hyperlipemia and ulcers (Xu 1995). Aqueous leaf extracts from *L. monopetalum* exhibited high radical scavenging activities against DPPH and superoxide radicals, related with the high phenolic content of the extracts, mainly in gallic and vanillic acids (Trabelsi et al. 2010). Gallic acid has *in vitro* anti-inflammatory, antimutagenic and antitumor properties, whereas vanillic acid possesses *in vivo* antiparasitic and *in vitro* antimicrobial (Ksouri et al. 2012). The flavonoid luteolin-7-O-β-glucoside is one of the major compounds found in *P. coronopus, P. lanceolata* and *P. major*, and has shown cytotoxic activities against human cancer cell lines, such as melanoma, renal and breast adenocarcinomas (Gálvez et al. 2003), possibly through the induction of DNA cleavage by topoisomerase I and II (López-Lázaro et al. 2002). The flavonoids rutin, apigenin, 3',6-dimethoxy apigenin and 4',7-dimethoxy apigenin have been isolated from *Teucrium polium* L. methanol extract due to their high antiradical activity on $DPPH^{\bullet}$ (Sharififar et al. 2009). This extract has shown protective effects on oxidative stress in murine models for diabetes type I and AD (Ksouri et al. 2012).

1.7. Halophytes from the Algarve Coast

Considerable halophyte biodiversity occurs in the Algarve coast with a manifold of potential biotechnological and therapeutic uses, but little research has been conducted to explore their phytopharmacological potential. The XtremeBio project (PTDC/MAR-EST/4346/2012), within which this PhD started, performed a phytochemical screening of more than 30 species regarding their chemical and nutritional profile, as well as diverse biological activities, namely *in vitro* antioxidant, anti-inflammatory, antitumoral, neuroprotective and antidiabetic. Results obtained with *Frankenia laevis* L., *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli, *Juncus acutus* L., *Juncus inflexus* L., *Juncus maritimus* L., *Limonium algarvense* Erben and *Polygonum maritimum* L. were particularly relevant. Therefore, this work focuses on deepening the knowledge on the potential biotechnological applications of the aforementioned species as sources of innovative high added-value bioactive natural products

with applications for the pharmaceutical, cosmetic and/or food industries. The following section will explore the information known about these species.

1.8. Plants used in this study

1.8.1. *Frankenia laevis*

Figure 1.10. Specimens of *F. laevis* in the field (A) and a close-up of the flowers (B) (photo by the author and from http://flora-on.pt).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Caryophyllales	Family: Frankeniaceae Genus: *Frankenia* Species: *Frankenia laevis* L.
Synonyms	-	
Common name	Sea heath (Portuguese: "urze do mar").	
Habitat	Coastal cliffs and sandy soils.	
Distribution	Mediterranean region, and along the Atlantic coast in Portugal, Spain and France, as well as in Azores islands (Brightmore 1979).	
Edibility	Information not found	
Medicinal uses	Used in Asian traditional medicine with astringent properties, as well as in the form of tinctures to treat diverse medical conditions, such as diarrhoea, dysentery, vaginal leucorrhoea, gonorrhoea, catarrh and mucous problems (Felter 1922).	
Biological properties	Essential oils have antibacterial activity against *Staphylococcus aureus, Micrococcus luteus* and *Salmonella typhimurium* (Saïdana et al. 2010). *In vitro* radical scavenging and copper chelating activities (Lopes et al. 2016).	
Bioactive compounds	Hexadecanoic acid, benzyl benzoate, benzyl cinnamate, farnesyl acetate, methyl linoleate, eugenol and β-caryophyllene were identified in the bioactive essential oils (Saïdana et al. 2010). Diverse phenolic sulphated compounds were also identified in whole plant aqueous-alcoholic extracts (Hussein 2004).	

*according to the checklist of "Flora de Portugal" (2010).

1.8.2. *Halopeplis amplexicaulis*

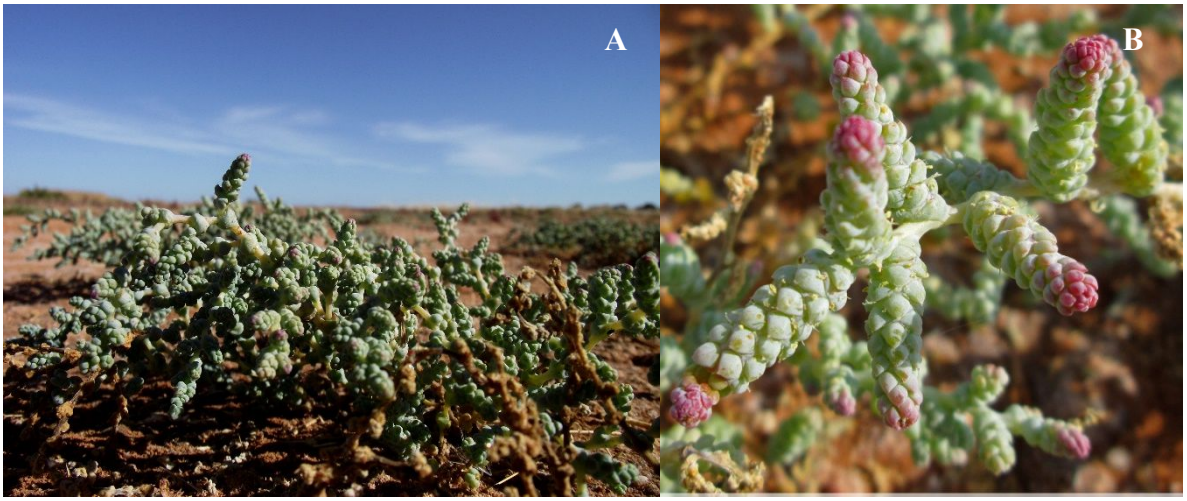

Fig. 1.11. Specimens of *H. amplexicaulis* in the field (A) and a close-up of the leaves (B) (photos by the author and from http://flora-on.pt).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Caryophyllales Family: Amaranthaceae Genus: *Halopeplis* Species: *Halopeplis amplexicaulis* (Vahl) Ces., Pass. & Gibelli
Synonyms	*Salicornia amplexicaulis*
Common name	-
Habitat	Brackish lagoons, inland or on the coast, and in the middle zone of salt marshes.
Distribution	Mediterranean region (Europe, Asia and North Africa) (Aukour, 2013). In the Iberian Peninsula it is only present on the coasts of the Algarve (Portugal), and Cádiz (Spain), and in continental lagoons of Zaragoza, Alicante, Malaga and Valencia (Spain) (Blanché and Molero, 1987).
Edibility	Edible in salads.
Medicinal uses	There are no records about medicinal uses of *H. amplexicaulis*, or of the genus.
Biological properties	Lopes et al. (2016) tested for *in vitro* antioxidant, metal chelating and anti-tyrosinase activities, but no activity was observed. No cytotoxicity was reported (Lopes et al., 2016).
Bioactive compounds	No bioactive compounds have yet been identified in this species.

*according to the checklist of "Flora de Portugal" (2010).

1.8.3. *Juncus acutus*

Fig. 1.12. Specimens of *J. acutus* in the field (A) and a close-up of the seeds (B) (photos by the author).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Juncales Family: Juncaceae Genus: *Juncus* Species: *Juncus acutus* L.
Synonyms	-
Common name	Spiny rush, sharp rush or sharp-pointed rush (Portuguese: "Junco-agudo").
Habitat	Saline swamps, banks of rivers, brackish lagoons, and occurs at the upper edge of salt marshes.
Distribution	Mediterranean, Europe, America, and temperate regions of South America, Africa and New Zealand.
Edibility	Information not found
Medicinal uses	Infused fruits mixed with barley grains were used for the treatment of colds in oriental medicine (El-Shamy et al. 2012).
Biological properties	*In vitro* anti-algal, anti-inflammatory, anti-eczematic, cytotoxic, anti-*Trypanosoma cruzi* activities (Dellagreca et al. 1992, 1993, 1998, 2002, 2003, 2005; Awaad 2006; Behery et al. 2007; Oliveira et al. 2016).
Bioactive compounds	Flavonoids, benzocoumarins, terpenes, sterols, phenolic acids, stilbenes, carotenoids, fatty acids, amino acids and phenanthrenes are the typical type of compounds found in this species (El-Shamy et al. 2012).

*according to the checklist of "Flora de Portugal" (2010).

1.8.4. *Juncus inflexus*

Fig. 1.13. Specimens of *J. inflexus* in the field (A) and a close-up of the seeds (B) (photos by the author).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Juncales Family: Juncaceae Genus: *Juncus* Species: *Juncus inflexus* L.
Synonyms	*Juncus glaucus*
Common name	Wire rush (Portuguese: "Junco-curvado" or "Junco-desmedulado").
Habitat	Ponds, wet meadows, saltmarshes and at the edges of freshwater.
Distribution	Native to Eurasia, North and South Africa. It was introduced into North and South America, Java, Australia and New Zealand.
Edibility	Information not found
Medicinal uses	Leaves are used in Basque country to treat warts and other skin diseases (Bús et al. 2018).
Biological properties	*In vitro* anti-microbial and antitumoral activities (Kuo et al. 2016; Tóth et al. 2016).
Bioactive compounds	Phenanthrenes (Kuo et al. 2016; Tóth et al. 2016).

*according to the checklist of "Flora de Portugal" (2010).

1.8.5. *Juncus maritimus*

Fig. 1.14. Specimens of *J. maritimus* in the field (A) and a close-up of the seeds (B) (photos by the author).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Juncales Family: Juncaceae Genus: *Juncus* Species: *Juncus maritimus* Lam.
Synonyms	-
Common name	Seaside rush (Portuguese: "Junco-das-esteiras" or "Junco-marítimo").
Habitat	Coastal salt marshes in the upper edge, saline meadows, and sand dunes.
Distribution	Mediterranean, Europe, America, and temperate regions of South America, Africa and New Zealand.
Edibility	Information not found
Medicinal uses	Rhizomes for insomnia (El-Shamy et al. 2012).
Biological properties	Anti-fungal (Sahli et al. 2018).
Bioactive compounds	Effusol (Sahli et al. 2018).

*according to the checklist of "Flora de Portugal" (2010).

1.8.6. *Limonium algarvense*

Fig. 1.15. Specimens of *L. algarvense* in the field (A) and after harvesting (B) (photos by the author).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Caryophyllales	Family: Plumbaginaceae Genus: *Limonium* Species: *Limonium algarvense* Erben
Synonyms	-	
Common name	Marsh rosemary or sea lavender (Potuguese: "ladina")	
Habitat	Coastal sand dunes and in the upper edge of salt marshes.	
Distribution	Endemic of the Iberian Peninsula southwest, from Algarve (Portugal) to Huelva and Cadiz (Spain) (Tutin et al. 1972; Franco 1984).	
Edibility	Information not found	
Medicinal uses	Infusions and decoctions were used for the treatment of diarrhoea, dysentery, mucous irritations, dyspepsia, pulmonary haemorrhage, laryngitis, bronchorrhea, catarrhal disorders, mouth and throat ulcers, gonorrhoea, leucorrhoea, anus and uterus prolapsus, and in some ophthalmic problems (Felter and Lloyd 1898).	
Biological properties	*In vitro* antioxidant (*L. echiodes*, *L. wrightii, L. tetragonum, L. gmelinii* and *L. brasiliense*), antimicrobial (*L. avei* and *L. stocksii*), antifungal (*L. echiodes* and *L. axillare*), anti-algal (*L. myrianthum*), cytotoxic (*L. axillare*), antitumoral *(L. sinense*), antiviral (*L. sinense*), and immunomodulatory (*L. sinense*) (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; Ali et al. 2013; Saïdana et al. 2013).	
Bioactive compounds	Sterols, phenolic compounds and polysaccharides were identified in *Limonium* genus (Kandil et al. 2000; Murray et al. 2004; Tang et al. 2012; Ali et al. 2013; Medini et al. 2014; Trabelsi et al. 2014).	

*according to the checklist of "Flora de Portugal" (2010).

1.8.7. *Polygonum maritimum*

Fig. 1.16. Specimens of *P. maritimum* in the field (A) and close-up of the leaves and flowers (B) (photos by the author and from http://flora-on.pt).

Taxonomic classification*	Kingdom: Plantae Phylum: Tracheophyta Class: Magnoliopsida Order: Caryophyllales	Family: Polygonaceae Genus: *Polygonum* Species: *Polygonum maritimum* L.
Synonyms	*Polygonum parviflorum, P. littorale, P. glaucum, P. chilense* and *Avicularia maritima*	
Common name	Sea knotgrass (Portuguese: "Polígono-marítimo").	
Habitat	Sandy coasts.	
Distribution	Europe, Mediterranean and Black Sea regions, Channel Islands, England and Belgium (Kilinc and Karaer 1995).	
Edibility	-	
Medicinal uses	For the treatment of dysentery, articular pain, and inflammation in China and Japan (Takasaki et al., 2001; Kawai et al., 2006; Fan et al., 2011), and for the treatment of diabetes in Europe, Africa and Asia (Soumyanath 2005; Bothon et al. 2013).	
Biological properties	*In vitro* antioxidant, antimicrobial and antitumoral activities (El-Haci et al. 2013; Jovanović et al. 2018).	
Bioactive compounds	Polygonocinol, (+)-8-hydroxycalamene, octacosyl, triacontyl ferulate, arylpropane, quercetin, quercitrin, catechin, sitosterol, gallic, ferulic, sinapic, caffeic and syringic acids, rutin, naringenin, epigallocatechin gallate, quercetin-3-O-galactoside, quercetin-3-O-glucoside, polygonophenone (Kazantzoglou et al. 2009; Jovanović et al. 2018; El-Haci et al. 2019).	

*according to the checklist of "Flora de Portugal" (2010).

1.9. Objectives of this study

Traditional medicine of different communities all over the world has acquired extensive knowledge about medicinal plants, sorting them in its different organs as treatments for different diseases, but little knowledge is available about halophytic plants (Tolossa et al. 2013). In the Algarve coast, there is a high diversity of halophytic species with a manifold of potential therapeutic uses. For example, in the areas surrounding the Ria Formosa lagoon, several species can be found belonging to different genera, namely *Limonium*, *Juncus*, *Frankenia*, *Halopeplis* and *Polygonum*. Information about the biological activities of the above-mentioned genera is scarce and some of the species, namely *H. amplexicaulis* and *L. algarvense* have never been tested for biological activities before. This work will contribute to raising knowledge on the potential application of underexploited halophytes abundant in the Algarve and/or of their extracts as a health-promoting commodity in the form of nutraceuticals, cosmetics, food additives and/or supplements.

In this sense, the main goals of this study are:

- Evaluate the *in vitro* antioxidant, neuroprotective, antidiabetic and anti-hyperpigmentation activities of extracts made from different anatomical organs of halophyte species from the Algarve coast;
- Characterize the phytochemical composition of the active extracts/fractions;
- Select the most promising species and test their sustainable cultivation;
- Elucidate the *in vitro* mechanism of action of juncunol, a selective antitumor molecule isolated from *J. acutus*.

1.10. References

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CHAPTER 2

SCREENING OF BIOLOGICAL ACTIVITIES

Abstract

Since ancient times, nature has been a source of inspiration for the development of new health-promoting commodities. For that, different approaches have been used to screen numerous biological samples for different biological targets associated with human pathologies, including for example neurodegenerative diseases, diabetes and cancer. The number of studies targeting natural products from halophytes is still reduced, when compared to those focusing on plants (glycophytes) and marine organisms, for example. In this context, the main aim of this chapter was to screen different anatomical organs from seven halophyte species from the Algarve coast [*F. laevis* (sea-heath), *H. amplexicaulis*, *J. acutus* (spiny rush), *J. inflexus* (wire rush), *J. maritimus* (seaside rush), *L. algarvense* (sea lavender) and *P. maritimum* (sea knotgrass)] for their *in vitro* antioxidant, neuroprotective, anti-hyperpigmentation and antidiabetic activities. The most promising extracts were selected and thoroughly investigated according to their biological properties: *Juncus sp.* for their promising *in vitro* antioxidant and anticholinesterase activities (chapter 3), sea lavender (chapter 4) and sea knotgrass (chapter 5) were selected for their strong antioxidant, neuroprotective, anti-hyperpigmentation and antidiabetic activities.

2.1. Introduction

Since ancient times, nature has been a source of medicines to treat different human ailments, which were at first based on practical and traditional aspects. Nowadays, due to the beneficial aspects of natural products, the screening of biological activities and identification/isolation of bioactive compounds from natural sources has a significant impact in several areas, since there is an increasing demand for novel compounds to provide healthcare assistance in diverse human conditions, such as inflammation, cancer, diabetes and neurological disorders (Balunas and Kinghorn 2005; Wang et al. 2007).

The drug discovery process usually combines different disciplines, as for example, biology, botany, ethnopharmacology, phytochemistry and biochemistry, and different approaches can be used. One model that is commonly used in the survey of natural products with biological activities involves the selection of the plants, followed by the extraction of natural products and screening for the selected biological activities, as for example antioxidant and anti-inflammatory. The plants' selection may comprise several approaches, including their traditional uses and biological properties already described in the species or genus, and the species abundance (Balunas and Kinghorn 2005). Moreover, different plant anatomical organs (*e.g.* flowers, peduncles, stems, leaves, roots, shoots) can be used, since diverse metabolites accumulate in different plant tissues and cells due to the different biochemical and physiological roles of each organ (Hutzler et al. 1998; Bedgood et al. 2005). Improved extraction of the bioactive compounds is usually achieved by the use of various solvents with different polarities (*e.g.* hexane, chloroform, dichloromethane, ethyl acetate, methanol, ethanol, or water), which promotes an increase in the number and diversity of extracted compounds, resulting in a more representative sample of the plant metabolites. However, the extraction effectiveness is also dependent on the species or biomass characteristics (*e.g.* location, season, plant organ, type of compounds) (Buhmann and Papenbrock 2013; Qasim et al. 2016).

The selection of the biological activities to be screened can also be made based on the traditional uses or reported properties of the target species, or by searching for alternative sources of compounds able to react with a known biological target (*e.g.* enzymes, cell receptors or ion channels), as well as according to the equipment infrastructures and the scientists' experience. The selected methods should fulfil several requirements, namely reproducibility, reduced costs, pharmacological relevance, validity, lack of ambiguity, accuracy, selectivity (to reduced false positives), and high sensitivity (allow testing of low concentrations of the bioactive compounds) (Vlietinck 1999; Hughes et al. 2011). These conditions are usually

fulfilled by *in vitro* models, which are therefore considered the most suitable for screening tests, and only after, the selected samples can undergo more complex testing models, such as cellular-based or *in vivo* ones (Vlietinck 1999; Hughes et al. 2011). Regardless of the selection reasons, the active extracts must undergo a chemical characterization by appropriate techniques [*e.g.* liquid (LC) or gas chromatography (GC) coupled to UV or mass spectrometry (MS) detectors] or may be fractionated and retested until the isolation of the active compound(s) is accomplished, followed by molecular structure elucidation and determination of its mechanism of action (Balunas and Kinghorn 2005).

In the present work, as detailed in the introduction section, the selection of the species was based on previous knowledge obtained from the XtremeBio project (PTDC/MAR-EST/4346/2012), which screened more than 30 halophyte species from the Algarve coast for relevant *in vitro* biological activities (antioxidant, anti-inflammatory, antitumoral, neuroprotective and antidiabetic). Most of the species belong to genera with reported important medicinal uses, and promising properties were found on the species *F. laevis, H. amplexicaulis, J. acutus, J. inflexus, J. maritimus, L. algarvense* and *P. maritimum.* This work focused on these halophytes and intended to perform an extensive screening of its biological activities, aiming at their biotechnological valorisation. For that purpose, different extracts (methanol and dichloromethane) were prepared from different anatomical organs and evaluated *in vitro* for radical scavenging activity (RSA) against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and nitric oxide (NO) radicals, ferric reducing antioxidant power (FRAP), and copper (CCA) and iron (ICA) chelating activities. The inhibitory capacity of the extracts was also appraised on enzymes implicated on the onset of neurodegenerative diseases, especially AD (AChE and BuChE), hyperpigmentation disorders (tyrosinase) and diabetes (α-amylase and α-glucosidase). The extracts exhibiting the most promising results were selected and further studied according to the bioactivity detected: the most promising samples were extracted using different solvents (*e.g.* ethanol, acetone, ethyl acetate, water) and methodologies (*e.g.* Soxhlet extraction, ultrasonic-assisted extraction, high-temperature extraction). Obtained extracts were evaluated *in vitro* for different biological activities, such as anti-wrinkles and anti-acne), by different assays, as for example enzyme and cell-based models. To identify the bioactive compounds (*e.g.* phenolics, phytosterols, fatty acids) the active extracts were chemically characterized using different methods, including as high-performance liquid chromatography (HPLC), gas chromatography and mass spectrometry (GC-MS), or ultra-high-resolution mass spectrometry (UHRMS) analyses.

2.2. Materials and methods

2.2.1. Chemicals

The DPPH and ABTS radicals, sodium nitrite, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), butylated hydroxytoluene (BHT), acetylcholinesterase from electric eel (EC 3.1.1.7), equine butyrylcholinesterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine iodide, 5-thio-2-nitrobenzoate (DTNB), tyrosinase from mushroom (EC 1.14.18.1), L-tyrosine, alpha-amylase from porcine pancreas (EC 3.2.1.1), alpha-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Germany). Lonza (Belgium) provided Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), trypsin, L-glutamine and penicillin/streptomycin. Additional reagents and solvents were obtained from VWR International (Belgium).

2.2.2. Plant material

Samples from *F. laevis, H. amplexicaulis, J. acutus, J. inflexus, J. maritimus L. algarvense,* and *P. maritimum* were collected in June of 2013, from different locations in the Algarve (coordinates: *F. laevis, J. acutus, J. inflexus,* and *J. maritimus,* 43°38'19.39" N 116°14'28.86" W; *H. amplexicaulis*, 43°38'19.39"N 116°14'28.86"W; *P. maritimum:* 37° 2' 33.079" N 7° 44' 47.321" W; *L. algarvense*, 37°2′6.526″N 7°58′ 58.465″W). The taxonomical classification was determined by the botanist Dr Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). When possible, plants were divided into their different anatomical organs [*F. laevis*: aerial parts (flowers + leaves + stems); *H. amplexicaulis*: aerial parts (leaves + stems); *L. algarvense:* flowers, peduncles and roots; *J. acutus* and *J. maritimus:* seeds, leaves and roots; *P. maritimum* and *J. inflexus:* leaves and roots], oven dried for 3 days at 50°C, powdered and stored at -20°C until needed.

2.2.3. Extraction

To increase the range of extractable compounds and the potential of extracting bioactive compounds, two different solvents of different polarities were used for extraction: one with

medium polarity (methanol, 0.762 E^N_T*) and one of lower polarity (dichloromethane, 0.309 E^N_T*). Thus, dried samples were separately mixed with methanol and dichloromethane (1:40, w/v), and extracted overnight at room temperature (RT), under stirring. Extracts were filtered (Whatman nº 4) and concentrated under reduced pressure at 40ºC, using a rotary evaporator. Dried extracts were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C at the concentration of 20 mg/mL.

2.2.4. Determination of antioxidant activity

The samples' activity was determined at 1 mg/mL, and when activity was higher than 50%, different concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were tested for determining the half-maximal concentration (IC_{50}) values (mg/mL). The absorbance was measured in a microplate reader (Biotek Synergy 4), and the activity was calculated as a percentage of inhibition, relative to a control containing DMSO in place of the sample.

2.2.4.1. 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) 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. The synthetic antioxidant butylated hydroxytoluene (BHT, E320), used as a preservative in food and cosmetics, was used as a positive control at the same concentrations of the samples. A colour control was used to prevent the influence of the extracts' colour in the assay, where the same volume of sample (22 µL) was mixed with 200 µL of methanol.

2.2.4.2. RSA on ABTS radical

The RSA on the 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. The samples (10 µL)

* E^N_T : Empirical solvent polarity parameter.

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. BHT was used as the positive control, at the same concentrations of the extracts.

2.2.4.3. RSA on nitric oxide (NO)

The NO scavenging activity was evaluated according to Baliga et al. (2003). The extracts (50 µL) were mixed in 96 well plates with 50 µL of 10 mM 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% HPO_3) were added and absorbance was read at 546 nm. N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as the positive control at the same concentrations of the extracts. The colour control consisted of 50 µL of each sample mixed with 100 µL of PBS.

2.2.4.4. 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), distilled water (50 µL) and 1% potassium ferricyanide (50 µL) were mixed in 96 well plates and incubated at 50ºC for 20 min. Then, 50 µL of 10% trichloroacetic acid (w/v, in water) and ferric chloride solution (0.1 %, w/v in water) were added, and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power, and results were expressed as a percentage of inhibition relative the positive control, BHT (at the concentration of 1 mg/mL).

2.2.4.5. Metal chelating activity on copper (CCA)

CCA was determined according to Megías et al. (2009), with a few modifications. Samples (30 µL) 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 $CuSO_4 \bullet 5H_20$ (50 µg/mL in water). The change in colour of the solution was measured at 632 nm. The synthetic metal chelator EDTA was used as a positive control at the same concentrations of the extracts. The colour control contained in 30 µL of each extract combined with 306 µL of the above-mentioned buffer.

2.2.4.6. Metal chelating activity on iron (ICA)

ICA was determined by measuring the formation of the Fe^{2+} ferrozine complex according to Megías et al. (2009), with some modifications. Samples (30 µL) were mixed in 96-well microplates with 200 µL of distilled water and 30 µL of a $FeCl_2$ 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 at 562 nm. EDTA was used as a positive control at concentrations from 0.06 to 1 mg/mL. The colour control comprised 30 µL of sample mixed 242.5 µL of the distilled water.

2.2.5. *In vitro* inhibition of enzymes related to neurological disorders

The samples' activity was determined as described in section 2.2.4.

2.2.5.1. AChE and BuChE inhibitory activities

The inhibitory effect of the extracts on AChE and BuChE was assessed according to Orhan et al. (2007). Samples (20 µL) were mixed with 140 µL of 0.02 M phosphate buffer (pH 8.0) and 20 µL of enzyme solution (0.28 U/mL in the same buffer). After an incubation period of 15 min. at 25ºC, 10 µL of the substrate (acetylcholine iodide or butyrylcholine chloride, 4 mg/mL in the previous buffer) and 20 µL of 5,5'-dithiobis-)2-nitrobenzoic acid) (DTNB, 1.2 mg/mL in ethanol) were added and incubated 15 min. at 25ºC. Then, absorbances were read at 412 nm. The clinically used cholinesterase inhibitor eserine (syn. physostigmine) was used as a standard at different concentrations (0.00001 - 1 mg/mL). The colour control consisted of 20 µL of the extracts mixed 190 µL of the referred buffer.

2.2.6. *In vitro* inhibition of enzymes related to hyperpigmentation

The samples' activity was determined as described in section 2.2.4.

2.2.6.1. Tyrosinase (TYRO) inhibitory activity

The inhibitory activity against TYRO was adapted from the method reported by Nerya et al. (2003), using L-tyrosine as a substrate. Samples (70 µL) were mixed with 30 µL of

enzyme solution (333 U/mL) in 25 mM potassium phosphate buffer (pH 6.5). After 5 min. of incubation at RT, 110 µL of L-tyrosine (2 mM in the previous buffer) were added and incubated for an additional 30 min. period, at RT. Then, the optical densities were read at 492 nm. Arbutin, a commercially available tyrosinase inhibitor, was used as the positive control at the same concentrations of the samples. The colour control contained 70 µL of the extracts and 140 µL of the referred buffer.

2.2.7. *In vitro* inhibition of key enzymes relevant for hyperglycaemia

The samples' activity was determined as described in section 2.2.4.

2.2.7.1. Alpha-amylase inhibitory activity

The α-amylase inhibitory activity was determined by the method described by Xiao et al. (2006). Samples (40 µL) were mixed in 96-well microplates with 40 µL of amylase solution (100 U/mL in 0.1 M sodium phosphate buffer, pH 7.0) and 40 µL of 0.1% starch solution (diluted in the previous buffer). After 10 min at 37ºC, 20 µL of 1M hydrochloric acid (HCl) and 100 µL of iodide solution (5 mM iodine (I_2) + 5 mM potassium iodide (KI), in distilled water) were added and the absorbance was measured at 580 nm. Acarbose, the active ingredient of a clinically used drug for controlling T2DM (Glucobay®), was used as the standard at concentrations between 0.2 and 10 mg/mL. The colour control comprised 40 µL of the extracts, 180 µL of the mentioned buffer, and 20 µL HCl.

2.2.7.2. Baker's yeast α-glucosidase inhibitory assay

The microbial (*Saccharomyces cerevisiae*) α-glucosidase inhibitory activity was determined according to the method described by Kwon et al. (2007). The extracts (50 µL) were mixed with 100 µL of enzyme solution (1.0 U/mL, in 0.1 M sodium phosphate buffer, pH 7.0), and incubated for 10 min. at 25ºC. Then, 50 µL of 5 mM p-nitrophenyl-a-D-glucopyranoside (NGP; diluted in the previous buffer) were added and incubated for an additional period of 5 min. at 25ºC. Absorbance was recorded at 405 nm. Acarbose was used as a positive control at concentrations from 0.2 to 10 mg/mL. The colour control consisted of 50 µL of the samples mixed with 100 µL of the same buffer.

2.2.8. Statistical analysis

Results were expressed as mean ± 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 the Tukey HSD test. *P* values lower than 0.05 were considered significant. All statistical analysis was performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation). The IC_{50} values were calculated by sigmoidal fitting of the data using the GraphPad Prism v. 5.0 program.

2.3. Results

2.3.1. *In vitro* antioxidant activity

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 (Badarinath et al. 2010; Niki et al. 2010). Thus, in this work, the extracts were evaluated by three *in vitro* radical-based assays (RSA on DPPH, ABTS and NO) (Table 2.1) as well as by three metal-related methods (FRAP and metal chelation of iron and copper) (Table 2.2).

Generally, the methanol extracts of leaves and roots of the sea knotgrass had the highest RSA on DPPH, exhibiting similar IC_{50} values (0.03 mg/mL), which were significantly lower than the one obtained with the positive control (BHT; 0.11 mg/mL). The methanol extract from flowers of the sea lavender also had a significant RSA towards the DPPH radical, with an IC_{50} of 0.09 mg/mL, statistically similar to those of the sea knotgrass leaves and roots and also lower than that of BHT ($P > 0.05$). Regarding the ABTS radical, the best results were observed in the methanol extracts from leaves and roots of the sea knotgrass, with IC_{50} values of 0.14 and 0.19 mg/mL, respectively, statistically similar to the IC_{50} value of BHT (0.14 mg/mL). Nevertheless, it is noteworthy the low IC_{50} value (0.27 mg/mL) of the methanol extract of the sea lavender flowers on the RSA of ABTS. Globally, extracts were more active against the DPPH than the ABTS radicals; none of the extracts was able to reduce the NO.

In general, the extracts showed higher CCA than ICA; in fact, only the dichloromethane extract of the sea heath had significant capacity to chelate iron. The highest capacity to chelate copper was obtained with the methanol extracts of the sea knotgrass leaves and of the sea lavender flowers, with similar IC_{50} values (0.29 mg/mL). Additionally, more than 81% of the samples were able to reduce iron, and this activity was, overall, more pronounced on the

methanol extracts. The best FRAP results were obtained with the methanol extracts from the sea lavender, namely flowers (IC_{50} = 0.01 mg/mL), roots (IC_{50} = 0.06 mg/mL) and peduncles (IC_{50} = 0.08 mg/mL). The sea knotgrass methanol extracts from leaves and roots had also high ability to reduce iron, with IC_{50} values of 0.05 and 0.06 mg/mL, respectively.

Table 2.1. Radical scavenging activity on DPPH, ABTS and NO radicals of methanol (MeOH) and dichloromethane (DCM) extracts of selected halophyte species. Results are expressed as IC_{50} values (mg/mL).

Species / samples	Common name	Plant Part	DPPH		ABTS		NO	
			MeOH	DCM	MeOH	DCM	MeOH	DCM
F. laevis	Sea heath	Aerial parts	0.25 ± 0.01^{bcde}	nr	0.65 ± 0.02^{h}	nr	na	na
H. amplexicaulis		Aerial parts	na	na	na	nr	na	na
J. acutus	Spiny rush	Seeds	0.39 ± 0.02^{defg}	0.44 ± 0.02^{efg}	0.54 ± 0.01^{fg}	0.48 ± 0.02^{ef}	na	na
		Leaves	0.32 ± 0.00^{cde}	0.43 ± 0.04^{efg}	0.63 ± 0.02^{h}	0.45 ± 0.01^{e}	na	na
		Roots	0.22 ± 0.01^{bcd}	0.18 ± 0.01^{abc}	0.35 ± 0.01^{d}	0.22 ± 0.01^{bc}	na	na
J. inflexus	Wire rush	Leaves	nr	nr	nr	nr	na	na
		Roots	0.72 ± 0.01^{ij}	0.52 ± 0.01^{fgh}	nr	0.93 ± 0.02^{j}	na	na
J. maritimus	Seaside rush	Seeds	0.17 ± 0.01^{abc}	nr	nr	nr	na	na
		Leaves	0.79 ± 0.00^{j}	0.68 ± 0.02^{hij}	nr	0.61 ± 0.02^{gh}	na	na
		Roots	0.57 ± 0.01^{ghi}	nr	0.90 ± 0.05^{j}	na	na	na
L. algarvense	Sea lavender	Flowers	0.09 ± 0.01^{ab}	na	0.27 ± 0.01^{c}	na	na	na
		Peduncles	0.37 ± 0.01^{cdef}	nr	0.80 ± 0.03^{i}	nr	na	na
		Leaves	0.54 ± 0.01^{fghi}	na	$0.97 \pm n.05^{j}$	nr	na	na
		Roots	0.23 ± 0.01^{bcd}	na	0.60 ± 0.03^{gh}	na	na	na
P. maritimum	Sea knotgrass	Leaves	0.03 ± 0.00^{a}	nr	0.14 ± 0.01^{a}	nr	nr	na
		Roots	0.03 ± 0.00^{a}	nr	0.19 ± 0.00^{ab}	nr	nr	na
BHT*			0.11 ± 0.01^{cdef}		0.14 ± 0.01^{a}		-	
L-NAME*			-		-		2.50 ± 0.01	

Values represent the mean ± SEM of at least three experiments performed in triplicate ($n = 9$). For each assay (DPPH, ABTS and NO) values followed by different letters are significantly different according to Tukey HSD test ($P < 0.05$). *positive controls; -: not tested; na: not active (< 10% of activity at 1 mg/mL); nr: IC_{50} not reached.

Table 2.2. Metal chelating activity on iron (ICA) and copper (CCA) and ferric reducing activity (FRAP) activity of methanol (MeOH) and dichloromethane (DCM) extracts of selected halophyte species. Results are expressed as IC_{50} values (mg/mL).

Species / samples	Common name	Plant Part	CCA		ICA		FRAP	
			MeOH	DCM	MeOH	DCM	MeOH	DCM
F. laevis	Sea heath	Aerial parts	0.78 ± 0.01^{e}	nr	nr	0.76 ± 0.05^{b}	0.51 ± 0.03^{i}	0.64 ± 0.02^{kl}
H. amplexicaulis		Aerial parts	nr	nr	nr	nr	nr	0.59 ± 0.01^{jk}
J. acutus	Spiny rush	Seeds	nr	nr	na	nr	0.29 ± 0.01^{gh}	0.19 ± 0.00^{e}
		Leaves	nr	nr	nr	nr	0.32 ± 0.01^{h}	0.25 ± 0.00^{fg}
		Roots	nr	nr	na	nr	0.23 ± 0.01^{ef}	0.17 ± 0.00^{de}
J. inflexus	Wire rush	Leaves	nr	nr	na	nr	nr	nr
		Roots	nr	nr	na	nr	0.94 ± 0.02^{n}	0.25 ± 0.01^{fg}
J. maritimus	Seaside rush	Seeds	0.63 ± 0.01^{d}	nr	nr	nr	0.58 ± 0.01^{j}	nr
		Leaves	nr	nr	nr	nr	0.65 ± 0.02^{l}	0.12 ± 0.00^{cd}
		Roots	nr	nr	nr	nr	0.33 ± 0.01^{h}	0.31 ± 0.00^{h}
L. algarvense	Sea lavender	Flowers	0.29 ± 0.01^{b}	na	nr	nr	0.01 ± 0.01^{a}	0.90 ± 0.01^{i}
		Peduncles	nr	nr	nr	na	0.08 ± 0.01^{bc}	nr
		Leaves	nr	nr	na	nr	0.18 ± 0.02^{e}	0.51 ± 0.01^{n}
		Roots	nr	na	nr	na	0.06 ± 0.01^{ab}	nr
P. maritimum	Sea knotgrass	Leaves	0.29 ± 0.01^{b}	nr	nr	nr	0.05 ± 0.00^{ab}	0.80 ± 0.04^{m}
		Roots	0.45 ± 0.01^{c}	nr	nr	nr	0.06 ± 0.00^{ab}	0.77 ± 0.05^{m}
EDTA*			0.17 ± 0.01^{a}		0.06 ± 0.00^{a}		-	

Values represent the mean ± SEM of at least three experiments performed in triplicate ($n = 9$). For each assay (CCA, ICA and FRAP) values followed by different letters are significantly different according to Tukey HSD test ($P < 0.05$). *positive control; -: not tested; na: not active (< 10% of activity at 1 mg/mL); nr: IC_{50} not reached.

2.3.2. *In vitro* neuroprotective activity

The evaluation of the *in vitro* neuroprotective potential of the extracts was assessed through their capacity to inhibit enzymes implicated in neurological disorders, especially AD, namely AChE and BuChE, and IC_{50} values are depicted in Table 2.3. Generally, the methanol extracts were more active than the dichloromethane ones. For instance, the methanol extracts of the sea knotgrass had the highest inhibitory activity towards AChE, particularly those from roots (IC_{50} = 0.17 mg/mL). Whereas, the methanol extracts from the sea lavender had the highest inhibition against BuChE (flowers: IC_{50} = 0.44 mg/mL; roots: IC_{50} = 0.43 mg/mL), followed by the methanol extracts of the sea knotgrass (leaves: IC_{50} = 0.62 mg/mL; roots: 0.61 mg/mL).

2.3.3. *In vitro* anti-hyperpigmentation activity

The evaluation of the anti-hyperpigmentation properties of the extracts was made by measuring their capacity to inhibit tyrosinase (TYRO), an enzyme implicated in the melanin biosynthesis, and the IC_{50} values are presented in Table 2.3. The methanol extract from flowers of the sea lavender had the highest inhibitory capacity, with an IC_{50} value of 0.11 mg/mL, which was significantly lower than the positive control (arbutin; IC_{50} = 0.17 mg/mL). The methanol extracts of the sea knotgrass also had a significant TYRO inhibition, with similar results for leaves and roots (leaves: IC_{50} = 0.60 mg/mL; roots: 0.59 mg/mL).

Table 2.3. AChE, BuChE and TYRO activities of methanol (MeOH) and dichloromethane (DCM) extracts of the selected halophyte species. Results are expressed as IC_{50} values (mg/mL).

Species / samples	Common name	Plant Part	AChE		BuChE		TYRO	
			MeOH	DCM	MeOH	DCM	MeOH	DCM
F. laevis	Sea-heath	Aerial parts	na	nr	nr	nr	nr	na
H. amplexicaulis		Aerial parts	na	nr	nr	nr	na	na
J. acutus	Spiny rush	Seeds	nr	nr	nr	nr	nr	nr
		Leaves	nr	nr	nr	0.81 ± 0.15^{c}	nr	na
		Roots	nr	nr	nr	nr	nr	nr
J. inflexus	Wire rush	Leaves	na	na	na	nr	nr	nr
		Roots	nr	nr	nr	nr	nr	nr
J. maritimus	Seaside rush	Seeds	na	na	na	na	nr	na
		Leaves	na	nr	na	nr	nr	nr
		Roots	na	nr	nr	nr	nr	nr
L. algarvense	Sea lavender	Flowers	nr	nr	0.44 ± 0.05^{b}	nr	0.11 ± 0.00^{a}	nr
		Peduncles	nr	nr	nr	nr	nr	na
		Leaves	nr	nr	na	nr	nr	na
		Roots	nr	nr	0.43 ± 0.01^{b}	na	0.72 ± 0.01^{d}	nr
P. maritimum	Sea knotgrass	Leaves	0.27 ± 0.01^{c}	0.91 ± 0.02^{d}	0.62 ± 0.03^{c}	nr	0.60 ± 0.01^{c}	na
		Roots	0.17 ± 0.01^{b}	nr	0.61 ± 0.03^{c}	nr	0.59 ± 0.00^{c}	nr
Eserine*			0.00003 ± 0.00^{a}		0.01 ± 0.00^{a}		-	
Arbutin*			-		-		0.17 ± 0.01^{b}	

Values represent the mean ± SEM of at least three experiments performed in triplicate ($n = 9$). For each assay (AChE, BuChE and TYRO) values followed by different letters are significantly different according to Tukey HSD test ($P < 0.05$). *positive controls; -: not tested; na: not active (< 10% of activity at 1 mg/mL); nr: IC_{50} not reached.

2.3.4. *In vitro* antidiabetic activity

Half-maximal inhibitory concentration values of the extracts with α-amylase and baker's yeast α-glucosidase inhibitory capacity are shown in Table 2.4. More than 80% of the extracts had strong α-glucosidase inhibition (81%), but only 6% of the samples could inhibit α-amylase (Table 2.4). The methanol extracts of the sea knotgrass had the highest inhibitory activity towards the baker's yeast α-glucosidase, with IC_{50} values of 0.02 and 0.03 mg/mL for roots and leaves, respectively. These values were around 100-fold more active than acarbose (IC_{50} = 3.14 mg/mL). Only the methanol and dichloromethane extracts from the seeds of seaside rush could inhibit α-amylase, particularly the methanol one with a statistically similar IC_{50} value to that of the positive control (IC_{50} = 7.80 mg/mL).

Table 2.4. Alpha-amylase and α-glucosidase inhibitory activities of methanol (MeOH) and dichloromethane (DCM) extracts of selected halophyte species. Results are expressed as IC_{50} values (mg/mL).

Species / samples	Common name	Plant Part	α-amylase		α-glucosidase	
			MeOH	DCM	MeOH	DCM
F. laevis	Sea-heath	Aerial parts	nr	nr	1.02 ± 0.01^{c}	0.52 ± 0.04^{bc}
H. amplexicaulis		Aerial parts	nr	nr	na	5.68 ± 0.30^{h}
J. acutus	Spiny rush	Seeds	nr	nr	nr	0.63 ± 0.03^{bc}
		Leaves	nr	nr	3.49 ± 0.26^{ef}	0.61 ± 0.01^{bc}
		Roots	nr	nr	1.48 ± 0.01^{d}	0.43 ± 0.02^{ab}
J. inflexus	Wire rush	Leaves	nr	nr	nr	4.77 ± 0.15^{g}
		Roots	na	nr	5.60 ± 0.13^{h}	0.76 ± 0.14^{bc}
J. maritimus	Seaside rush	Seeds	7.80 ± 0.29^{a}	8.83 ± 0.37^{b}	na	4.94 ± 0.49^{g}
		Leaves	nr	nr	na	3.81 ± 0.35^{f}
		Roots	na	nr	nr	0.57 ± 0.06^{bc}
L. algarvense	Sea lavender	Flowers	nr	nr	0.53 ± 0.01^{bc}	0.64 ± 0.02^{bc}
		Peduncles	nr	nr	1.61 ± 0.02^{d}	0.62 ± 0.00^{bc}
		Leaves	nr	nr	6.19 ± 0.12^{i}	0.67 ± 0.01^{bc}
		Roots	nr	nr	0.87 ± 0.01^{bc}	0.60 ± 0.00^{bc}
P. maritimum	Sea knotgrass	Leaves	nr	nr	0.03 ± 0.00^{a}	0.59 ± 0.03^{bc}
		Roots	nr	nr	0.02 ± 0.00^{a}	0.63 ± 0.01^{bc}
Acarbose*			7.80 ± 0.10^{a}		3.14 ± 0.13^{e}	

Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). For each assay (α-amylase and α-glucosidase) values followed by different letters are significantly different according to Tukey HSD test ($P < 0.05$). *positive controls; -: not tested; na: not active (< 10% of activity at 10 mg/mL); nr: IC_{50} not reached.

2.4. Conclusions and directions for the following chapters

The screening for the *in vitro* antioxidant, neuroprotective, antidiabetic and anti-hyperpigmentation potential of seven halophyte species resulted in a success rate of 43%, *i.e.*, extracts that showed activity above 50% in at least one assay. *Juncus* species showed high antioxidant and anti-cholinesterase inhibitory properties. In turn, the sea lavender and the sea knotgrass had high radical scavenging, iron reduction and metal chelation activities, as well as anti-cholinesterase and -tyrosinase properties; the sea knotgrass also had strong α-amylase and α-glucosidase inhibition. Therefore, these species were selected and further evaluated for biological and chemical properties, which will be presented and fully discussed in chapters 3 to 5, as detailed below.

Chapter 3 – Biological activities and chemical composition of *Juncus* sp. This chapter focused on the *in vitro* antioxidant and neuroprotective properties of *Juncus* species (spiny rush and seaside rush*:* seeds, leaves and roots; wire rush*:* leaves and roots), followed by the isolation and identification of the bioactive compound (juncunol, Rodrigues et al. 2017b). This chapter also includes a preliminary evaluation of juncunol's mode of action (Rodrigues et al. 2018b), which was previously isolated from the aerial parts of the spiny rush*,* regarding its cytotoxic and selective activity towards hepatocellular carcinoma cells (Rodrigues et al. 2014).

Chapter 4 – Biological activities and chemical composition of *Limonium algarvense*. This chapter aimed at unravelling the possible biotechnological applications of the sea lavender. For that purpose, different extracts were prepared from its anatomical organs and evaluated for *in vitro* antioxidant properties and chemical composition by high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) (Rodrigues et al. 2015). Since flowers exhibited the highest antioxidant activity, they were further explored for *in vitro* antioxidant and anti-inflammatory properties. For that purpose, infusions and decoctions were prepared, which are the traditionally used formulations for sea lavenders (*Limonium sp.*). Green tea was used for comparison due to its recognized health-promoting properties (epidemiological studies and clinical trials; Rodrigues et al. 2016). Infusions and decoctions obtained from herbal mixtures containing different ratios of sea lavender flowers' biomass and green tea were also prepared and assessed for *in vitro* antioxidant, neuroprotective and antidiabetic properties (Rodrigues et al. 2019a). Finally, a procedure for the greenhouse cultivation of the sea lavender was tested using saline irrigation of aquaculture wastewater. In this work, the influence of the irrigation salinity was evaluated on plant growth performance and on the *in vitro* antioxidant properties of produced biomass (Rodrigues et al. a, submitted for publication).

Chapter 5 – Biological activities and chemical composition of *Polygonum maritimum*. In this chapter the *in vitro* antioxidant, anti-inflammatory, antidiabetic and neuroprotective properties of the sea knotgrass were explored, followed by chemical profiling of the bioactive extracts (Rodrigues et al. 2017, 2018a). This species was also appraised for *in vitro* anti-hyperpigmentation properties and other possible skincare applications (Rodrigues et al. 2019b). A cultivation procedure for the sea knotgrass was then tested, in greenhouse conditions, where different salinity irrigation conditions were used along with a multi-harvest regime. The influence of these agronomic conditions was evaluated on the plants' growth performance, and on the *in vitro* antioxidant, anti-inflammatory and chemical profiles of extracts from produced biomass (Rodrigues et al. 2019c; Rodrigues et al. in press).

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CHAPTER 3

BIOLOGICAL ACTIVITIES AND CHEMICAL COMPOSITION OF *JUNCUS SP.*

Rodrigues MJ, Katkam GN, Zengin G, Mollica A, Varela J, Barreira L, Custódio L (2017). Juncaceae species as sources of innovative bioactive compounds for the food industry: *In vitro* antioxidant activity, neuroprotective properties and *in silico* studies. Food Chem Toxicol, 107, 590-596.

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Juncaceae species as sources of innovative bioactive compounds for the food industry: *In vitro* antioxidant activity, neuroprotective properties and in silico studies

Maria João Rodrigues [a], Katkam N. Gangadhar [a, d], Gokhan Zengin [b], Adriano Mollica [c], João Varela [a], Luísa Barreira [a], Luísa Custódio [a, *]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] Selcuk University, Science Faculty, Department of Biology, Campus, 42250 Konya, Turkey
[c] Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, 66100 Chieti, Italy
[d] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da Republica, Oeiras P-2780-157, Portugal

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ABSTRACT

Several *Juncus* species are traditionally used as sedative and to treat health problems like insomnia. This work was based on the hypothesis that *Juncus acutus, J. maritimus* and *J. inflexus* may have molecules with bioactivities relevant for the improvement of cognitive functions and thus with potential use as food additives and/or nutraceuticals. Therefore leaves and roots extracts of those species were evaluated for radical scavenging (RSA) and metal chelating activities, and for *in vitro* inhibition of acetyl-(AChE) and butyrylcholinesterase (BuChE). The bioactive compound was isolated and identified by HPLC-DAD, and its anticholinesterase capacity was determined by different assays. Docking studies were performed to elucidate its inhibitory mechanism. The dichloromethane root extract of *J. acutus* had the highest RSA against DPPH and ABTS radicals, and the dichloromethane extract of *J. maritimus* leaves had the uppermost FRAP. The dichloromethane extract from *J. acutus* leaves had the strongest BuChE inhibition. Juncunol was the bioactive compound, exhibiting dual anticholinesterase capacity on enzyme-based assays and AChE inhibition in neuronal and glial cells *in vitro*. Molecular docking studies indicate juncunol as a competitive reversible inhibitor. Our results suggest that *Juncus* spp. can be sources of bioactive compounds with application in the food industry as cognitive-enhancer nutraceuticals.

1. Introduction

The *Juncus* genus is the most represented of the Juncaceae family comprising more than 300 species distributed amongst all continents and being mainly found in salt marshes and badly-drained soils (El-Shamy et al., 2015). Several *Juncus* species are used in traditional medicine as sedative and to treat different health disorders, as for example insomnia (El-Shamy et al., 2015). These health benefits can be ascribed to the presence of different classes of compounds such as flavonoids, coumarins, terpenes, sterols, phenolic acids, stilbenes and phenanthrenes (El-Shamy et al., 2015), the latter being typically found in this genus (Kovács et al., 2008).

Europe is facing an aging population problem and it is estimated that a quarter of the European citizens will be over 60 years old in 2020 (WHO, 2011). As the population ages brain disorders such as dementia become an increasing burden. Dementia is characterized by the loss of memory, thinking impairment, behavioural alterations and loss of ability to perform daily living activities. Alzheimer's disease (AD) is the most common type of dementia; it has no cure and is the fourth main death cause in developed countries after cancer and cardiovascular diseases (WHO, 2014). AD is a multifactor neurodegenerative disorder biochemically characterized by decreased levels of acetylcholine (ACh), a neurotransmitter hydrolysed by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Thus, inhibition of AChE and/or BuChE results in an increase of ACh levels thereby improving cognitive functions (Greig et al., 2005). Oxidative stress is also associated with the development and progression of different forms of

* Corresponding author. Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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dementia. The brain has a high lipidic content, a high oxygen consumption and reduced antioxidant defences. As result, reactive oxygen species (ROS) are constantly formed by excitatory amino acids and neurotransmitters contributing to oxidative stress states and causing injuries to neuronal and glial cells (Uttara et al., 2009). Transition metals such as iron and copper also accept or donate electrons that induce ROS formation, contributing to oxidative stress and disease progression (Chen et al., 2012). In this sense, compounds with antioxidant and metal chelation capacities are considered effective in the prevention of brain oxidative damage and neuronal loss associated with neurodegeneration (Uttara et al., 2009).

There is a growing consumer's trend to use natural products, like those aiming to improve cognitive functions, and different nutra- and pharmaceutical products have been developed using plant-derived materials both from edible (e.g. *Ginkgo biloba, Melissa oficinallis, Panax ginseng* and *Valeriana officinalis*) and non-edible materials (e.g. *Physostigma venenosum, Atropa belladonna*) (Wilkinson et al., 2002; Kennedy and Wightman, 2011; Atanasov et al., 2015). Evidence shows that the use of different herbal products can result in cognitive enhancement in patients with mild-to-moderate AD through, for example, the inhibition of cholinesterases and protection against oxidative stress (Jivad and Rabiei, 2014). Chemical and biological studies on plants commonly used as folk medicine are thus relevant aiming to identify new sources of innovative products and/or molecules to be used in the food industry as cognitive enhancer supplements (Kennedy and Wightman, 2011).

Juncaceae species are used as a source of therapeutic compounds in traditional medicine. In this sense we hypothesized that *Juncus acutus, J. maritimus* and *J. inflexus* may contain molecules with biological activities relevant for the improvement of cognitive functions and thus with potential use in the food industry as food additives and/or nutraceuticals. To test this hypothesis, leaves and roots of the above mentioned species were extracted with methanol and dichloromethane and evaluated for their antioxidant properties and capacity to inhibit enzymes related with neurodegeneration, namely AChE and BuChE. The most bioactive extract was submitted to a bioguided fractionation, the bioactive compound (juncunol) was isolated and identified and its cholinesterase inhibitory capacity was evaluated through enzyme- and cell-based assays. Molecular modelling studies were also carried to elucidate juncunol's mode of action. Several studies are available in literature regarding botanical bioactive compounds with neuroprotective properties (e.g. Rocha et al., 2017; Zengin et al., 2017 Grochowski et al., 2017). However, to the best of our knowledge this is the first report of the *in vitro* neuroprotective features of species belonging to the *Juncus* genus and of juncunol. Besides deepening the knowledge about these plants, our results can also contribute to the valorisation of Juncaceae species as novel sources of bioactive compounds to be used in the food industry as cognitive enhancer nutraceuticals.

2. Material and methods

2.1. Chemicals

The 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), butylated hydroxytoluene (BHT), acetylcholinesterase (EC 3.1.1.7), butytylcholinesterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine iodide and 5-thio-2-nitrobenzoate (DTNB) were purchased from Sigma-Aldrich (Germany). Remaining reagents and solvents were acquired from VWR International (Belgium).

2.2. Plant material and extraction

Samples from *J. acutus* L., *J. maritimus* Lam. and *J. inflexus* L. were collected in South 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 voucher specimens are kept in the herbarium of the MarBiotech laboratory (voucher codes MBH23: *J. acutus*, MBH24: *J. maritimus*; MBH25: *J. inflexus*). Plants were divided in leaves and roots, oven dried for 3 days at 40 °C, powdered and stored at −20 °C until needed. Dried samples were separately mixed with methanol and dichloromethane (1:40, w/v), and extracted overnight at room temperature (RT) under stirring. Extracts were filtered (Whatman nº 4) and concentrated under reduced pressure. Dried extracts were dissolved in dimethylsulfoxide (DMSO) at the concentration of 10 mg/mL and stored (−20 °C).

2.3. Determination of antioxidant activity by radical-based assays

2.3.1. Radical scavenging activity (RSA) on DPPH, ABTS and NO radicals

The RSA was evaluated as described before (Custódio et al., 2015a) on samples at concentrations ranging from 6 to 1000 μg/mL. Results were expressed as percentage of inhibition (%) relative to a control containing DMSO instead of the sample and as half maximal inhibitory concentration (IC_{50} values, μg/mL). BHT (DPPH and ABTS assays) and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, NO assay) were used as a positive controls at the same concentrations of the extracts.

2.4. Determination of antioxidant activity by metal-related methods

2.4.1. Ferric reducing antioxidant power (FRAP)

The ability of the extracts (at concentrations from 6 to 1000 μg/mL) to reduce Fe^{3+} was assayed according to Megías et al. (2009). Increased absorbance of the reaction mixture indicated increased reducing power. Results were expressed as inhibition (%) relative to the positive control (BHT) at 1 mg/mL and as IC_{50} values (μg/mL).

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

CCA and ICA were determined according to Megías et al. (2009) on samples at different concentrations (6–1000 μg/mL). EDTA was used as a positive control at the concentration of 1000 μg/mL and results were expressed as inhibition (%) relative to a control containing DMSO.

2.4.3. In vitro inhibition of AChE and BuChE

Enzymatic inhibition was assessed according to Custódio et al. (2015b) on samples at different concentrations (40–1000 μg/mL). Results were expressed as inhibition (%) relative to a control containing DMSO and as IC_{50} values (μg/mL). Galanthamine was used as standard at the same concentrations of the samples.

Bioguided fractionation, isolation of the bioactive compound and high-performance liquid chromatography (HPLC) analysis.

The dichloromethane extract of *J. acutus* aerial part (5 g) was subjected to a bioguided fractionation through a silica gel (120 mesh) column chromatography (25 cm × 2 cm i.d) (Rodrigues et al., 2014). Fractions with similar thin layer chromatography (TLC) profiles were mixed, resulting in 11 fractions (F1-11) that were tested for AChE and BuChE inhibitory activity as described previously. Based on the results, fraction **F2** (0.76 g) was selected and subjected to a new silica (60 mesh) column chromatography (14 cm × 2 cm i.d) and sequentially eluted with hexane and a

mixture of hexane and ethyl acetate (98:2 and 95:5) affording a pure compound, which was stored at −20 °C until further use.

For identification purposes the isolated pure compound was dissolved in dichloromethane at the concentration of 10 mg/mL and analysed by HPLC-DAD (Knauer Smartline, Germany) constituted by the following modules: vacuum degasser (E4320V2), quaternary pump (EA4300V1) and the diode array detector (E4350). Data acquisition and instrumental control were performed by the software Clarity (v2.6.04.402). Analyses were performed on a Luna 5u C18 (2) 100A, 250 × 4.6 mm, 5 μm particle size (Phenomenex, Spain). The mobile phase consists on a mixture of acetonitrile (solvent A) and mili-Q water with the following gradient: 0–30 min: 80-10% A, 30–35 min: 10-0% A, 35–45 min: 0-0% A, 45–50 min: 80% A, using a flow of 1.0 mL/min. The injection volume was 20 μL and the detector was set at 216 nm. For identification, the retention parameters of each assay were compared with pure juncunol and the peak purity with the UV-visible spectral reference data. The samples were filtered through a 0.2 μm membrane syringe filter prior analysis.

2.5. Anticholinesterase inhibitory capacity of juncunol

2.5.1. Enzyme-based assay

Inhibitory effect of juncunol at concentrations ranging from 300 to 5000 μM was evaluated on AChE and BuChE by the method described previously. Results were expressed as inhibition (%) relative to a control containing DMSO and as IC_{50} values (μg/mL). Galanthamine was used as standard at concentrations between 0.3 and 10 μM.

2.5.2. Cell-based assay

Inhibitory capacity of juncunol on AChE and BuChE was further evaluated using neuroblastoma (SH-SY5Y) and murine microglia (N9) cell lines, which were respectively obtained from Dr. Eduardo Soriano (Barcelona Science Park, Spain) and from Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal). SH-SY5Y and N9 cells were maintained as described elsewhere (Rodrigues et al., 2014; Custódio et al., 2015b). To select non-toxic concentrations (viability ≥ 80%), cells were plated at a density of 2×10^4 cells/well in 96-well tissue microplates, incubated overnight and juncunol was applied at concentrations up to 1000 μM for 2 h. Control cells were treated with DMSO at the highest concentration used in test wells (0.5%) and cell viability was determined by the MTT assay (Custódio et al., 2015b). Results were expressed in terms of cell viability (%). The AChE and BuChE inhibition of non-toxic concentrations of juncunol (up to 200 μM) was then evaluated on SH-SY5Y and N9 cells by the DTNB assay (Racchi et al., 2001; Santillo and Liu, 2015). In brief, cells were plated at a density of 2×10^4 cells/well in 96-well tissue microplates, incubated overnight and treated with selected concentrations of juncunol for 2 h. Then the extracts and culture medium were removed and 140 μL of 0.1 mM sodium phosphate buffer (pH 8.0) were added to each well. Enzymatic inhibition was determined by the method described previously. Results were expressed as inhibition (%) relative to a control containing DMSO and as IC_{50} values (μM). Galanthamine was used as standard at the same concentrations of juncunol.

2.6. Molecular modeling

2.6.1. Enzymes and ligand preparation

Molecular modelling studies were carried out to elucidate the inhibitory activity of juncunol towards AChE and BuChE. The crystallographic enzyme structures have been downloaded from the Protein Databank RCSB PDB (Berman et al., 2000): AChE (pdb:4X3C) (Pesaresi) in complex with tacrine-nicotinamide hybrid inhibitor and BuChE (pdb: 4BDS) (Nachon et al., 2013) in complex with tacrine. Enzymes have been prepared for docking by the Swiss-PDB viewer (Guex and Peitsch, 1997) to correct the errors in the crystal structures and then finalized by Wizard Preparation tool embedded in Maestro 10.0.6 (Release, 2015). Juncunol structure was built by Maestro, neutralized at neutral pH and minimized. Then the ligand was used in the docking experiments without further modifications.

2.6.2. Docking experiments

Docking experiments were carried by Gold suite 6 software (Verdonk et al., 2003) using the scoring function *GoldScore* as previously reported (Atanasova et al., 2015; Mocan et al., 2016a, 2016b). The docking grid has been automatically calculated in a radius of 10 Å from the center of the crystallographic ligand. The best docking pose found for juncunol in each enzyme-juncunol complex is depicted in Fig. 1.

2.7. Statistical analysis

Results were expressed as mean ± 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 the Tukey HSD test ($P < 0.05$). Statistical analysis were performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation). IC_{50} 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

Neurodegeneration is linked to ROS-mediated brain damage and leads to neuronal cells death (Uttara et al., 2009). Accumulation of redox metals in the brain, such as iron and copper, also promotes ROS generation through the Haber-Weiss/Fenton reaction (Koppenol, 2001). Thus, the use of antioxidants is considered a potential therapeutic approach in the prevention and/or treatment of neurodegenerative diseases due to their ability to neutralize free radicals and/or to their capacity to bind and neutralize metal ions (Uttara et al., 2009). In this work extracts from *Juncus* species were generally effective as DPPH and ABTS scavengers and iron reducers but had no significant capacity to scavenge the NO radical. In the DPPH assay the dichloromethane extract of *J. acutus* roots allowed for the lowest IC_{50} value (178 μg/mL), statistically similar ($p < 0.005$) to the one obtained with the positive control (BHT; $IC_{50} = 111$ μg/mL; Table 1). The dichloromethane extract of *J. acutus* roots also had a strong ABTS antiradical activity with an IC_{50} value of 221 μg/mL, comparable to BHT (139 μg/mL). This result is in accordance with previous findings made by other authors, where a high RSA against ABTS was reported for different compounds isolated from methanol extracts from *J. acutus* rhizomes (e.g. luteolin; Behery et al., 2013). In addition, methanol and ether extracts of *J. acutus* aerial organs were already described to have a significant DPPH and ABTS antiradical activities (Rodrigues et al., 2014). The dichloromethane leaf extract of *J. maritimus* was the most effective in the FRAP assay ($IC_{50} = 125$ μg/mL). Accordingly, a high FRAP was previously observed in methanol extracts from other Juncaceae species, as for example *J. effuses* (Gan et al., 2010). Overall, our results indicate that methanol and dichloromethane extracts from leaves and roots from *J. acutus* and leaves from *J. maritimus* contain molecules able to scavenge free radicals, namely DPPH and ABTS, and also to reduce Fe^{3+}. Considering this, these species may be a

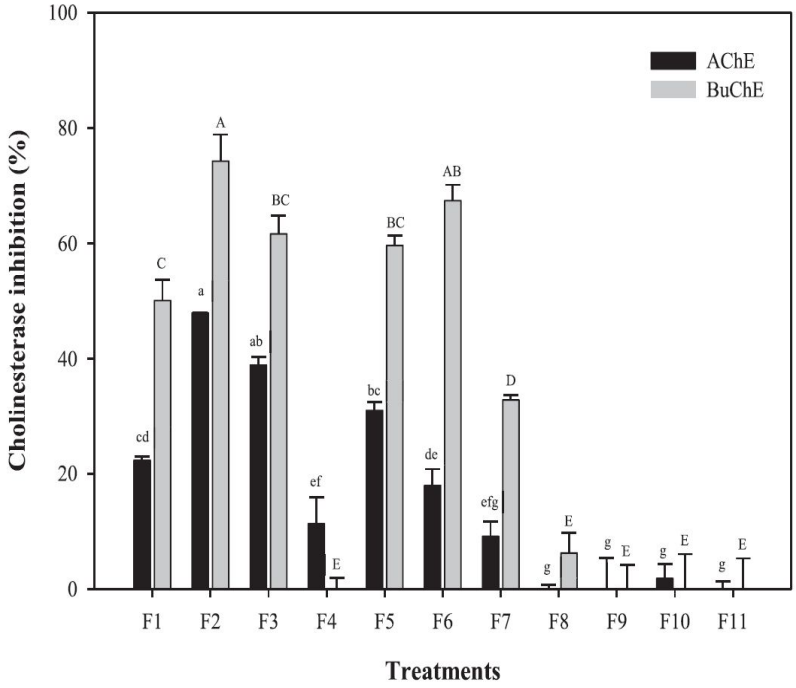

Fig. 1. Cholinesterase inhibitory activities of the fractions obtained from the *J. acutus* leaves, and eserine (standard), on AChE (AChE; black bars) and BuChE (grey bars). Results were expressed as inhibition (%) relative to the negative control, at the concentration of 1000 μg/mL. Bars followed by different letters (a-h for black bars and A-E for grey bars) are significantly different (Tukey HSD test; $P < 0.05$).

Table 1
Radical scavenging activity (RSA) on DPPH and ABTS radicals, and ferric reducing antioxidant power (FRAP), expressed as half-minimal inhibitory concentration (IC_{50}; μg/mL), of the methanol (MeOH) and dichloromethane (DCM) extracts of leaves and roots of *J. acutus*, *J. maritimus* and *J. inflexus*.

Species/Compound	Organ	DPPH		ABTS		FRAP	
		MeOH	DCM	MeOH	DCM	MeOH	DCM
J. acutus	Leaves	316 ± 4^{c}	426 ± 39^{d}	628 ± 17^{d}	453 ± 11^{c}	320 ± 11^{c}	247 ± 3^{abc}
	Roots	223 ± 6^{b}	178 ± 6^{ab}	348 ± 11^{b}	221 ± 5^{a}	226 ± 9^{abc}	173 ± 4^{ab}
J. maritimus	Leaves	793 ± 4^{g}	679 ± 17^{f}	>1000	610 ± 16^{d}	649 ± 18^{d}	125 ± 1^{a}
	Roots	573 ± 9^{e}	>1000	903 ± 46^{e}	>1000	325 ± 11^{c}	310 ± 3^{bc}
J. inflexus	Leaves	>1000	>1000	>1000	>1000	>1000	>1000
	Roots	720 ± 8^{fg}	520 ± 14^{e}	–	930 ± 18^{e}	941 ± 24^{e}	255 ± 6^{abc}
BHT*		111 ± 9^{a}		139 ± 11^{a}		–	–

Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). For the same method, values followed by different letters (a-g for DPPH, and a-e for ABTS and FRAP) are significantly different according to the Tukey HSD test ($P < 0.05$). *: positive control.

promising source of useful compounds that can be used as novel food supplements for the prevention of oxidative-stress age-related degenerative brain disorders. Moreover, to the best of our knowledge, this is the first report of the antioxidant potential of *J. maritimus*.

3.2. AChE and BuChE inhibition

The reduction of ACh levels is associated with the progression of different neurological disorders including AD. Since ACh is hydrolysed by the enzymes AChE and BuChE, their inhibition is a valuable tool to improve cognitive functions (Greig et al., 2005). In this work extracts were tested at the concentration of 1000 μg/mL for their capacity to inhibit AChE and BuChE and the IC_{50} values were determined when more than 50% of activity was achieved. Results are summarized on Table 2. The dichloromethane leaf extract from *J. acutus* was the only with anticholinergic potential, with IC_{50} values of 665 μg/mL and 951 μg/mL towards BuChE and AChE, respectively. Usually, plant extracts have a higher capacity to inhibit AChE than BuChE (Rocha et al., 2017; Grochowski et al., 2017; Zengin et al., 2017). However, approximately 10–15% of the human cholinergic neurons such as those located in the hippocampus and amygdala express BuChE instead of AChE in their cell bodies and proximal dendrites (Furukawa-Hibi et al., 2011). In the healthy human brain AChE and BuChE are present in the proportion of 4:1 but this ratio can be significantly modified in the brain of AD patients since the AChE activity can drop to 45% during disease progression as a consequence of neurons and axons loss, and the BuChE activity can increase up to 2-fold (Furukawa-Hibi et al., 2011). BuChE selective inhibitors or dual inhibitors of AChE and BuChE are able to efficiently increase the levels of the synaptic ACh (Greig et al., 2005). The dichloromethane extract from *J. acutus* leaves can be considered a promising source of molecules and/or products with anticholinesterase activity, especially against BuChE, and thus with the potential to improve cognitive functions. In fact, there are several reports of the anticholinesterase capacity of

Table 2
BuChE inhibitory activities of the methanol (MeOH) and dichloromethane (DCM) extracts of leaves and roots of *J. acutus*. Results are expressed as IC_{50} values (μg/mL).

Species/Compound	Organ	BuChE	
		MeOH	DCM
J. acutus	Leaves	>1000	665 ± 78^{b}
	Roots	>1000	951 ± 68^{c}
Galanthamine*		0.32 ± 0.01^{a}	

Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate ($n = 9$). Values followed by different letters (a-c) are significantly different according to the Tukey HSD test ($P < 0.05$). *: positive control.

extracts from different plant species and their use as food supplements to reduce age-related memory loss (e.g. *G. biloba*, De Feudis and Drieu, 2001; Wettstein, 2000). Moreover, to the best of our knowledge, this is the first report about the cholinesterase inhibitory activity of Juncaceae species suggesting their potential use as innovative additives with cognitive improvement capacity in the food industry.

3.3. Isolation and identification of the bioactive compound

The dichloromethane leaf extract from *J. acutus* was submitted to a bio-guided fractionation resulting in 11 fractions. Fractions **F1**, **F2**, **F3**, **F5** and **F6** had potent BuChE inhibition (>50%) at the highest concentration tested (1000 μg/mL), while **F2**, **F3** and **F5** had moderate AChE inhibition (30–50%; Fig. 1). **F2** allowed the highest ChE-I (BuChE: 74.3%; AChE: 48.8%; Fig. 2) and thus it was further fractionated until an active and apparently pure molecule was detected by TLC and HPLC analysis. Chemical analysis was made by HPLC/DAD and the identification was performed by comparison of the retention parameters and the UV-visible spectral data with standard juncunol (1,7-dimethyl-5-vinyl-9,10-dihydrophenanthren-2-ol), confirming this molecule as the active compound (Rodrigues et al., 2014).

3.4. Anticholinesterase activity of juncunol

Similarly to the results obtained with the crude extract, juncunol had a higher activity against BuChE ($IC_{50} = 758$ μM) than AChE ($IC_{50} = 940$ μM) and was more active than the crude active extract

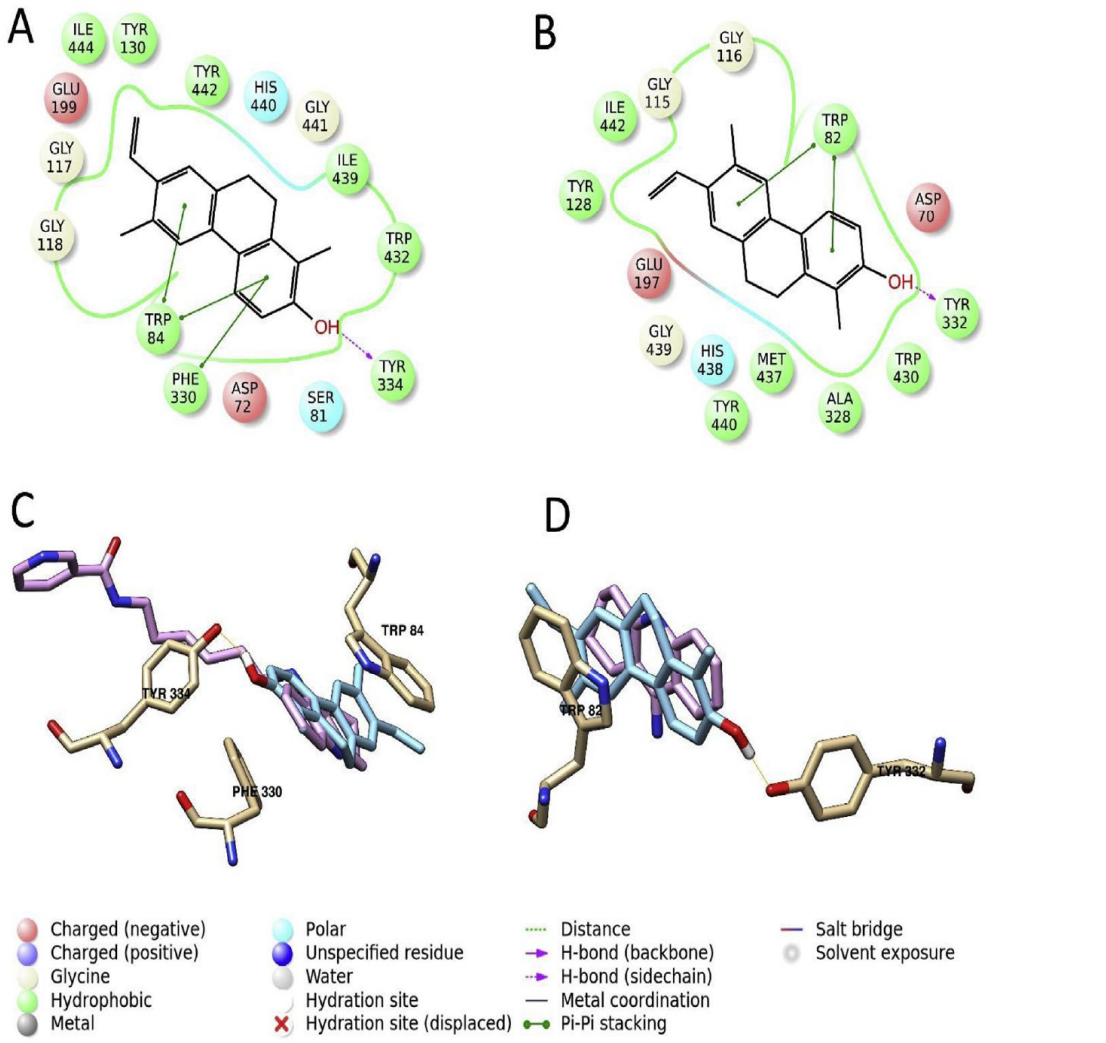

Fig. 2. Representation of the interaction network found for the lowest energy pose of juncunol docked to (A) AChE and (B) BuChE. (C) Crystallographic inhibitor tacrine-nicotinamide hybrid (pink) superimposed to juncunol (cyan) docked to AChE; (D) crystallographic inhibitor tacrine (pink) superimposed to juncunol (cyan) docked to BuChE. Hydrogen bonds are depicted as orange lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
AChE and BuChE inhibitory activities of juncunol and galanthamine (positive control). Results are expressed as IC_{50} values (μM).

Compound	Enzyme-based assay		Cell-based assay	
	AChE	BuChE	AChE (SH-SY5Y)	AChE (N9)
Juncunol	940 ± 10^{b}	758 ± 2^{a}	158 ± 1^{b}	117 ± 2^{b}
Galanthamine*	27.2 ± 0.7^{a}	1110 ± 0.05^{b}	4566 ± 825^{a}	1730 ± 31^{a}

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 (a-b) are significantly different according to the Tukey HSD test ($P < 0.05$). *: positive control. SH-SY5Y: human neuroblastoma cell line; N9: murine microglia cell line.

(Table 3). In the cell based-systems juncunol was able to significantly inhibit the activity of AChE with IC_{50} values of 158 μM (SH-SY5Y) and 117 μM (N9), which were 6–8 times lower than those obtained in the enzyme-based assay (Table 3). It was not possible to evaluate the BuChE inhibitory capacity of juncunol since in our experiment conditions the level of the latter enzyme was not detectable. BuChE is found in much lower concentrations in the brain and has a more restricted distribution than AChE, thus making it more difficult to detect (Mesulam and Giacobini, 2000). The outcomes from cell-based assays are generally more physiologically relevant than those from cell-free assay systems (Burroughs et al., 2012). In this context, our results suggest for the first time that juncunol can significantly inhibit AChE and BuChE and thus may have cognitive enhancement properties. Hence, juncunol should be further explored for its potential as a health-promoting supplement in the food industry.

3.5. Molecular docking

Computational techniques are successfully applied in the study of natural products to predict the ligand-target interaction mode, relative affinity and to better understand the molecular basis of their action against key enzymes. The molecular modelling carried out for juncunol on AChE and BuChE revealed that the compound fits well in the enzymatic pocket of both enzymes and it is capable to establish a network of interactions with the amino acid residues present in the cavity. The structure of juncunol is remarkably similar in terms of size and structure to that of tacrine, which is the crystallographic ligand present as standard inhibitor in both the enzymatic Xray structures used in this study. The best pose found for juncunol has been superimposed to the crystallographic inhibitor in the enzymatic cavity of the enzyme (Fig. 2). For AChE the best pose is stabilized by two π-π interactions with Trp84, one π-π interaction to Phe330, and one hydrogen bond to Tyr334. The best ranked pose of juncunol docked to BuChE is stabilized, similarly, by the two π-π interactions to Trp82 and one hydrogen bond to Tyr332. The most relevant structural difference with tacrine, narciprimine and other commercial inhibitors, e.g. galantamine, is the absence of the nitrogen atoms in the tricyclic nucleus of juncunol. The nitrogen atoms present in the above-mentioned compounds are responsible for key interactions with the enzymes. In juncunol this absence is compensated by the presence of the phenolic group. In fact, in the lowest energy pose found by molecular modeling, the tricyclic moiety is superimposed to that of tacrine, whereas the hydroxyl group of juncunol is involved in a key interaction with the residues Tyr334 and Tyr332 of AChE and BuChE respectively, by the formation of hydrogen bond. Our results indicate that juncunol may act as a competitive reversible inhibitor, confirming its potential application as cognitive enhancer.

4. Conclusions

This study reports for the first time the potential cognitive improvement potential of leaves and roots of *J. acutus*, *J. maritimus* and *J. inflexus* through their capacity to inhibit enzymes related with the onset of neurological disorders (AChE and BuChE) and antioxidant potential. The dichloromethane extract from *J. acutus* leaves had a high BuChE inhibitory activity and thus was fractionated until the bioactive compound was isolated and identified as juncunol. Juncunol had dual anti-cholinesterase capacity on enzyme-based assays and was able to significantly inhibit the activity of AChE on neuronal and glial cells *in vitro*, possibly through a competitive reversible inhibition. Our results strongly suggest that the Juncaceae species included in this study can be sources of bioactive compounds to be used as food aditives and/or targeting the preparation of innovative nutraceuticals with cognitive improvement properties.

Conflicts of interest

The authors declare no conflicts of interest.

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Transparency document

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Short communication

In vitro and *in silico* approaches to unveil the mechanisms underlying the cytotoxic effect of juncunol on human hepatocarcinoma cells

Maria João Rodrigues[a], Catarina Vizetto-Duarte[a], Katkam N. Gangadhar[a], Gokhan Zengin[b], Adriano Mollica[c], João Varela[a], Luísa Barreira[a], Luísa Custódio[a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Faro, Portugal
[b] Selcuk University, Science Faculty, Department of Biology, Campus, Konya, Turkey
[c] Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy

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ABSTRACT

Background: Juncunol is a phenanthrene isolated from the halophyte species *Juncus acutus*, with selective cytotoxic activity towards human hepatocarcinoma (HepG2) cells. However, its mechanism of action is unknown.
Methods: The *in vitro* cytotoxic mechanism of juncunol was evaluated on HepG2 cells through several methods to elucidate its potential to induce apoptotic features, decrease mitochondrial membrane potential, promote internal ROS production and influence cell cycle. We also report its haemolytic activity on human erythrocytes and *in silico* DNA-binding studies.
Results: Juncunol induced an increase in the number of apoptotic cells in a concentration-dependent manner, accompanied by a decrease in the mitochondrial membrane potential. No significant differences were observed in production of reactive oxygen species (ROS). Moreover, juncunol application at the IC_{50} value significantly induced cell cycle arrest in the G0/G1 phase comparatively to the control group. No significant haemolysis was detected. *In silico* studies indicate that juncunol seems to bind between GC base pairs.
Conclusion: Juncunol reduced HepG2 cells proliferation through the induction of apoptotic cellular death, in a concentration-dependent manner. Apoptosis induction seems to be related with a decrease of the mitochondrial membrane potential but not with ROS production. Juncunol had no haemolytic activity and may act as a DNA intercalator. Our data suggests juncunol as a suitable candidate for more detailed studies, including *in vivo* experiments, in order to completely characterize its mode of action.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide. It is considered as a primary malignancy of the liver and occurs mainly in people with chronic liver diseases, such as cirrhosis or hepatitis [1]. This is a particular aggressive type of cancer characterized by a high metastatic potential and resistance to cytotoxic agents [1] and, thus, there is an urgent need to identify new leads for HCC chemotherapeutics.

The chemical diversity and structural complexity of natural products make them ideal candidates for the identification of novel chemotherapeutics and a high number of such phytochemicals are at the origin of several drugs in clinical use for the treatment of cancer, as for example paclitaxel (Taxol®) and vimblastine (Velban®). Moreover, different medicinal plants display chemopreventive effects on tumour cell lines, including HepG2 cells [2]. On a previous work, juncunol was isolated and identified from the halophyte species *Juncus acutus* as a cytotoxic and selective molecule towards the human hepatocellular carcinoma cell line HepG2 [3]. The current work aimed to provide a first insight on the molecular mechanisms underlying this effect through different techniques, namely flow cytometry analysis, annexin V/propidium iodide (PI), dichlorofluorescein, rhodamine 123 and PI cell staining, allowing the evaluation of apoptotic features, internal reactive oxygen species (ROS) production, mitochondrial membrane potential and cell cycle disruption, respectively. We also report the haemolytic activity of juncunol and *in silico* DNA-binding studies.

Materials and methods

Rhodamine 123 (Rh123), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate

* Corresponding author.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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Table 1
Effect of juncunol on apoptosis induction, mitochondrial membrane potential alteration (Δψm) and ROS production by HepG2 cells.

Compound	Apoptotic cells (%)	Loss of Δψm (%)	ROS (%)
DMSO*	23.0 ± 1.2^{c}	8.30 ± 6.40^{c}	0.70 ± 0.09^{a}
Juncunol			
14 μM	62.8 ± 0.7^{b}	24.1 ± 0.5^{bc}	3.60 ± 1.03^{a}
18 μM	66.4 ± 2.4^{ab}	29.4 ± 0.4^{b}	3.98 ± 0.50^{a}
23 μM	73.0 ± 0.9^{a}	39.5 ± 1.2^{ab}	3.60 ± 1.92^{a}
Etoposide**	72.8 ± 0.7^{a}	47.4 ± 0.9^{a}	0.99 ± 0.18^{a}

Values represent the mean ± standard error of the mean (SEM) of at three determinations ($n = 3$). For the same method, values followed by different letters (a–c) are significantly different according to the Tukey HSD test ($p < 0.05$).
* Negative control, 0.5%.
** Positive control.

(H_2DCFDA) were purchased from Sigma-Aldrich (Germany). Lonza (Belgium) provided Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), trypsin, L-glutamine and penicillin/streptomycin. Additional reagents and solvents were obtained from VWR International (Belgium). Juncunol was isolated and purified as described in Rodrigues et al. [3] and applied at the IC_{50} concentration (18 μM), which was previously determined by the MTT assay, ±25% (14 and 23 μM). HepG2 cells (human hepatocellular carcinoma) were kindly provided by Dr. Vera Marques and maintained routinely as described before [3]. Negative and positive controls consisted of culture medium with 0.5% DMSO and etoposide (2.4 μM) respectively. Flow cytometry studies were performed in a FACS Calibur Flow Cytometer (Becton-Dickinson, East Rutherford, NJ, USA) plotting at least 20 000 events per sample.

Measurement of annexin V binding

Cells were seeded in 6-well plates at 1×10^6 cells/mL, incubated overnight and treated with juncunol for 72 h. Measurement of phosphatidylserine redistribution in a plasma membrane was conducted by the Annexin V-FITC Apoptosis Detection kit according to the manufacturer's instructions (Abnova Corporation, Taiwan). Cells were analysed by flow cytometry.

Cell cycle detection

Cells (5×10^5 cell/mL) were plated on 96-wells plates, incubated overnight, treated for 72 h with juncunol and processed for cell cycle analysis according to Vizetto-Duarte et al. [1]. DNA histograms were analysed by flow cytometry.

Measurement of mitochondrial membrane potential (ΔΨm)

Cells were plated in 6-well plates at 5×10^5 cells/mL, incubated overnight and then treated with juncunol for 72 h. Cells were then trypsinised, processed according to Wu et al. [4] and analysed by flow cytometry.

Intracellular ROS determination

Cells were seeded in 6-well plates (5×10^5 cells/mL), incubated overnight and treated with juncunol for 72 h. Cells were then processed according to Wu et al. [4] and analysed by flow cytometry.

In vitro *haemolytic activity*

Haemolytic activity of juncunol was determined according to Oliveira et al. [5]. Results were expressed as a percentage of haemolysis compared with the positive control (milli-Q water: 100% haemolysis).

In silico *DNA-binding assays*

The ds-DNA (CGATCG)2 in complex with ellipticine (pdb: 1Z3F) has been downloaded from the PDB database [6]. The ligand and the 6-dp-DNA were separated and the water molecules and other molecules were removed. The ds-DNA was prepared according to previously described methods [7]. Juncunol and the crystallographic ligand ellipticine were prepared by ligand preparation suite embedded in Maestro 2015-2. The pH was set at 7.4 and the protonation state was calculated by Ionizer using the OPLS3 force field. The prepared structures and chemical structures are depicted in Fig. S1.

Statistical analysis

Results were expressed as mean ± 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 the Tukey HSD test ($p < 0.05$). Statistical

Table 2
Effect of juncunol treatment on the HepG2 cells cycle progression.

Compound	Cell cycle phase		
	G0/G1 (%)	S (%)	G2/M (%)
DMSO*	65.8 ± 0.6^{b}	20.6 ± 0.3^{ab}	11.9 ± 0.1^{b}
Juncunol			
14 μM	71.1 ± 0.4^{a}	15.1 ± 1.2^{c}	12.6 ± 1.4^{b}
18 μM	71.4 ± 0.8^{a}	14.3 ± 0.4^{c}	12.8 ± 1.1^{b}
23 μM	69.4 ± 0.7^{ab}	17.0 ± 1.3^{bc}	10.6 ± 1.2^{b}
Etoposide**	21.4 ± 0.8^{c}	23.2 ± 0.5^{a}	49.2 ± 1.0^{a}

Values represent the mean ± standard error of the mean (SEM) of at three determinations ($n = 3$). For each cell cycle phase, values followed by different letters (a–c) are significantly different according to the Tukey HSD test ($p < 0.05$).
* Negative control (0.5%).
** Positive control (2.4 μM).

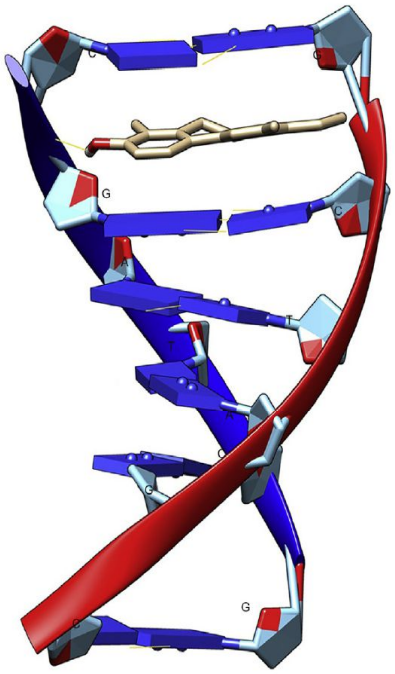

Fig. 1. Best pose of Juncunol docked between CG base pair on the ds-DNA fragment (CGATCG)2, PDB: 1Z3F.

analysis was performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation).

Results and discussion

Apoptosis induction by juncunol

Apoptosis induction in cancer cells is one of main anticancer strategies and a common mechanism of action of several anticancer drugs [1]. To evaluate if juncunol was able to induce apoptosis, HepG2 cells were stained with annexin V-FITC and propidium iodide (PI) and analysed by flow cytometry. Annexin V binds to the phosphatidylserine that turns over to the outer side of the plasma membrane leaflet of the apoptotic cells, whereas PI binds to nucleic acids of the dead cells [1].

Treatment of HepG2 cells with juncunol at 14, 18 and 23 μM resulted in an increase of apoptotic cells to 62.8 ± 0.7, 66.4 ± 2.4 and 73 ± 0.9%, respectively, comparatively to the control (DMSO: 23 ± 1.2%; Table 1, Fig. S2), thus suggesting that apoptosis contributed significantly to the reduction of cellular viability. This is in accordance with findings on other phenanthrenes such as denbinobin, fimbriol B and 2,3,5-trihydroxy-4,9-dimethoxyphenanthrene, which have shown strong apoptosis induction capacity on hepatic cells [8].

Juncunol-induced apoptosis through loss of Δψm

Apoptotic pathway is associated with alterations in the Δψm which leads to mitochondrial membrane permeabilization, release of cytochrome c and caspase activation [9]. Treatment of HepG2 cells with juncunol resulted in a significant ($p < 0.05$) concentration-dependent loss of Δψm, as shown by the progressive decrease in the fluorescence intensity (FL-1) (Table 1, Fig. S3). This activity was also reported for other phenanthrenes such as triptolide which induced apoptosis through a decrease in the Δψm in liver and cervical cells [10,11].

Juncunol-induced apoptosis through redox modifications

Mitochondria are pivotal for ROS formation during cellular respiration [12]. Mitochondrial dysfunction causes energy impairment and oxidative stress, leading to the apoptotic process initiation. In some cases, alterations in the mitochondria are mediated by oxidation-reduction status, including the increase in intracellular ROS levels [13]. In this work, none of the applied concentrations of juncunol induced a significant ROS generation ($p > 0.05$; Table 1), thus suggesting that juncunol-induced apoptosis is not mediated by ROS production.

Cell cycle arrest induced by juncunol

The application of chemotherapeutic agents usually contributes to cell cycle arrest at specific stages, decreasing the tumour cells' growth and proliferation and inducing apoptosis [14]. Application of juncunol at the IC_{50} concentration resulted in a significant increase ($p < 0.05$) of the G0/G1 stage cells (71.4 ± 0.8%) comparatively to the control group (65.8 ± 0.6%), and a decrease of the S phase (14.3 ± 0.4%) compared to the control group (20.6 ± 0.3%) (Table 2). Different phenanthrenes also influence cell cycle, as for example cryptotanshinone and tylophorine which induced HepG2 cell cycle arrest at the G1 phase [15,16], while the application of tanshinone on human colon cancer cells resulted in G0/G1 arrest [17].

In vitro *haemolytic activity*

Drug induced haemolysis is a frequent complication associated with cancer chemotherapy that results from the interaction of the drug with the erythrocyte membrane, leading to cell lysis [18]. This condition has diverse consequences, as for example jaundice, reticulocytosis and haemolytic anemia. [19]. In this work, no significant haemolysis was observed after application of juncunol ($p > 0.05$; Table S1), suggesting its low toxicity to cells *in vivo* [5].

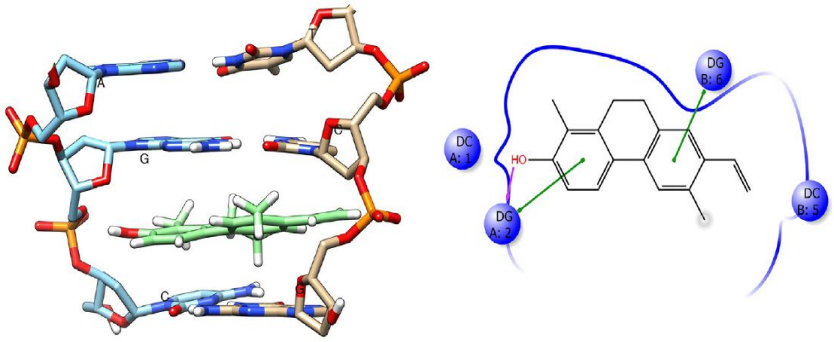

Fig. 2. Induced Fit Docking best pose and its interactions in the binding pocket.

In silico *DNA-binding assays*

Ligands with cytotoxic activity frequently have the ability to bind the ds-DNA, which may intercalate between base pairs. One of the most common binding modes of small aromatic molecules, such as juncunol, is intercalation. To evaluate this hypothesis, docking studies were performed using rigid ds-DNA and a flexible binding domain (induced fit docking) (Figs. 1 and 2).

The standard fragment $(CGATCG)_2$ co-crystallized with ellipticine as intercalating agent was self-docked by Glide SP, and XP and Autodock Vina [20]. Glide failed in repositioning the crystallographic ligand, whereas Autodock Vina was capable to correctly insert ellepticine in the original position with a RMSD = 0.5 A. Therefore, Autodock vina was used to carry out the docking experiment of juncunol in the crystallographic ds-DNA. The docking grid was manually centred at the ellipticine and the DNA strain was kept rigid whereas the ligand is considered flexible. Vina was able to find 8 poses and the best pose had a docking score of −8.2 (Fig. 2). Juncunol was docked similarly to the crystallographic ligand, possessing a similar structure to ellipticine in terms of stereoelectronic distribution with three electron-rich portions, two aromatic and a double bond, capable to establish pi–pi stacks with the ds-DNA base pairs. Moreover, the hydroxylic group of the phenolic moiety represents a further interaction of juncunol to the DNA strain.

Ligands binding usually induce conformational changes in the active site. The docking grid centre was specified from the ligand bound to the DNA strain and the cubic grid had a side length of 10 Å. A 2.5 kcal/mol energy window was used for ligand conformational sampling. The scaling factors to soften the potentials of the receptors and ligands were set to 0.5 in both cases. A maximum of 20 poses were saved. All residues within 5.0 Å of ligand poses were refined using the Prime molecular dynamics module allowing the binding domain flexibility. Glide XP was used for the re-docking step into the top 20 receptor structures generated within 30 kcal/mol of the best structure by the Prime refinement. The best ranked pose is depicted in Fig. 2 together with the 2D representation of the pose interactions.

Juncunol has a peculiar dehydrophenantrenic nucleus that gave a flat three-dimensional structure typical of ds-DNA intercalators, corroborated by the computational experiments. Indeed, juncunol seems to bind between GC base pairs similarly to the natural compound ellipticine.

Conclusions

Our results indicate that the cytotoxic activity of juncunol on HepG2 cells occurs through apoptosis induction related with a reduction of the mitochondrial membrane potential but not with ROS production, and may act as a DNA intercalator. As juncunol had no significant haemolytic activity, it could be as suitable candidate for more detailed studies regarding its antitumoral potential.

Conflicts of interest

The authors have declared that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pharep.2018.02.001.

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SUPPLEMENTARY MATERIAL

Table S1. Haemolytic effect of juncunol on human red blood cells (RBCs). Juncunol was applied at different concentrations (14, 18 and 23 µM) for 1h. DMSO (0.5%) was used as the negative control, and etoposide (2.4 µM) for comparison purposes.

Compound	Concentration	Haemolysis (%)
DMSO	0.5%	4.29 ± 0.48[a]
Juncunol	14 µM	4.02 ± 0.18[a]
	18 µM	3.59 ± 0.06[a]
	23 µM	4.11 ± 0.57[a]
Etoposide*	2.4 µM	3.46 ± 0.15[a]

Values represent the mean ± standard error of the mean (SEM) of at three determinations (n = 3). Values followed by different letters (a-b) are significantly different according to the Tukey HSD test ($p < 0.05$). *: positive control.

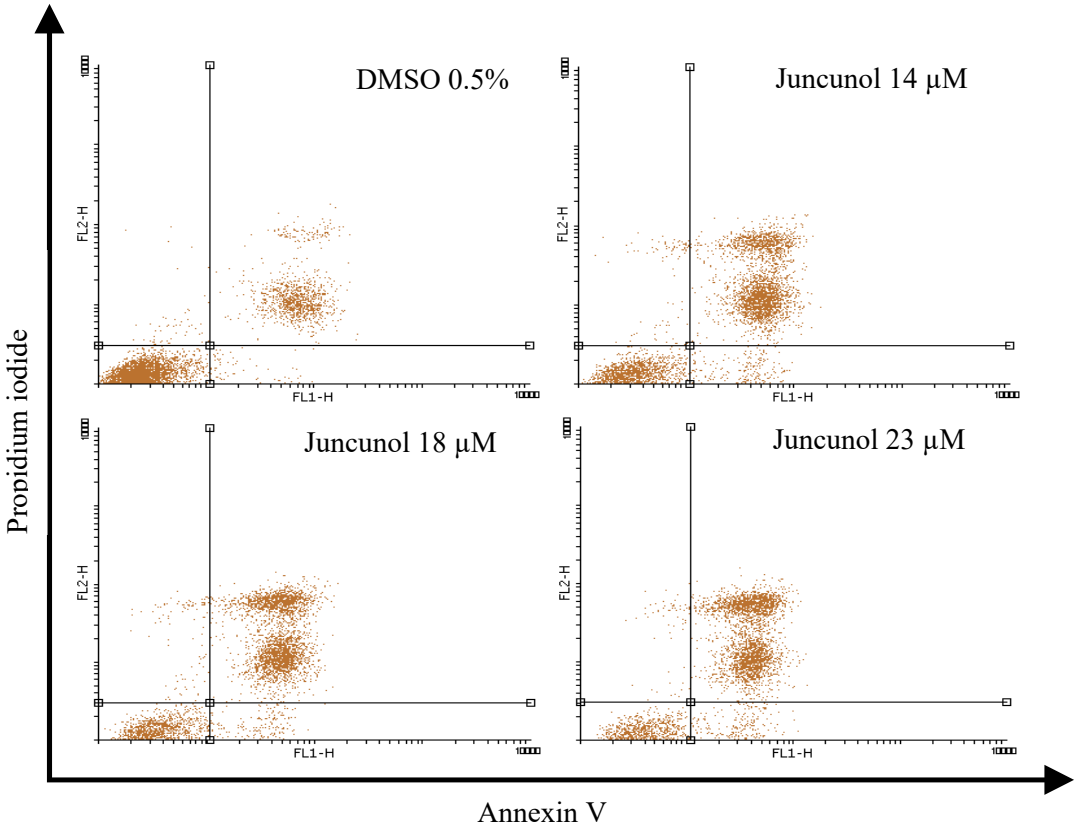

Fig. S1. Chemical structure of juncunol and ellipticine (present in the crystal structure PDB:1Z3F) and their 3D conformation.

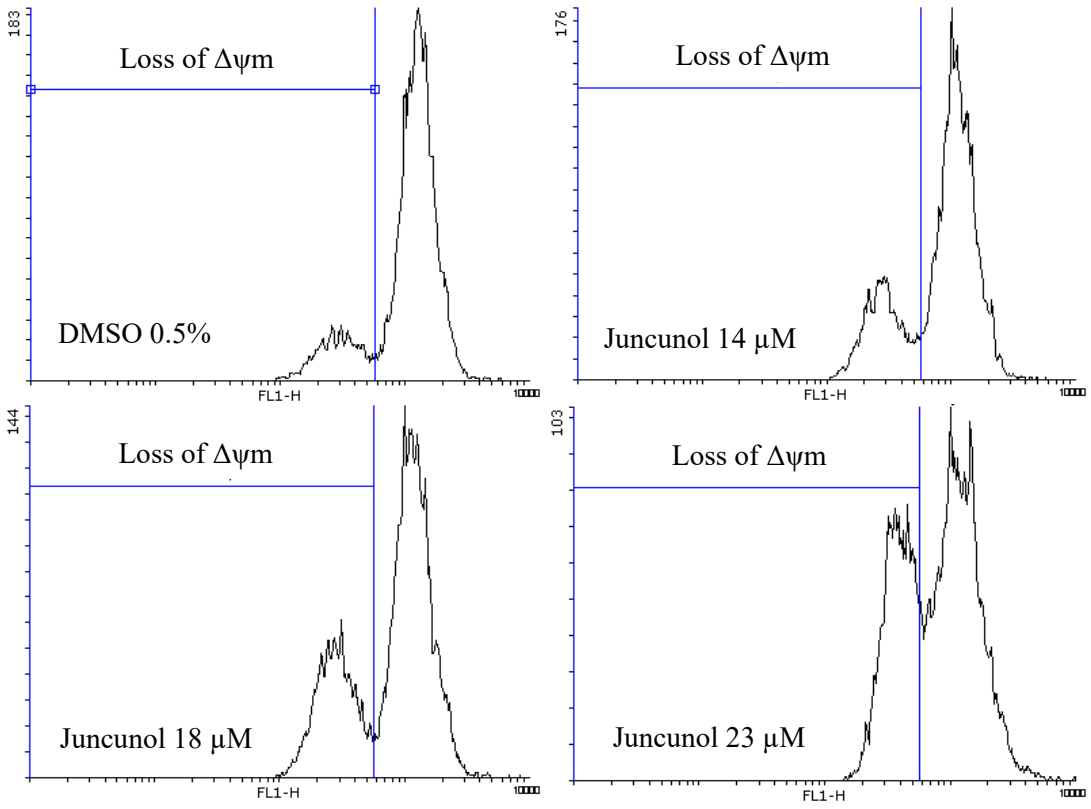

Fig. S2. Induction of apoptosis by juncunol in HepG2 cells. They were treated with DMSO (0.5%), and juncunol at different concentrations (14, 18 and 23 µM) for 72h, double stained with annexin V-FITC and propidium iodide (PI), and analysed by flow cytometry. The lower and upper right represent early (Annexin+/PI-) and late apoptotic (Annexin+/PI+) cells, respectively, whereas the upper left quadrant represents the necrotic cells (Annexin-/PI+).

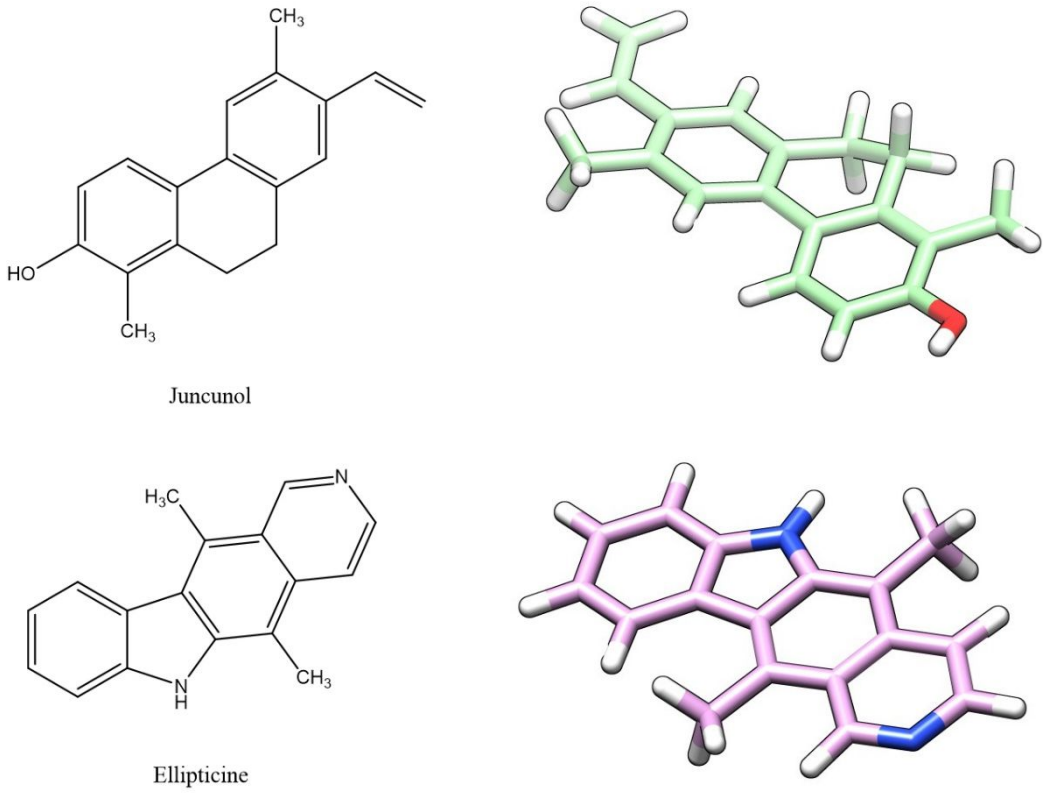

Fig. S3. Effect of juncunol on the alteration of mitochondrial transmembrane potential (Δψm) in HepG2 cells. Mitochondrial membrane potential was measured by flow cytometry with Rh123 staining after a treatment with DMSO (0.5%) and different concentrations of juncunol (14, 18 and 23 µM) for 72h.

CHAPTER 4

BIOLOGICAL ACTIVITIES AND CHEMICAL COMPOSITION OF *LIMONIUM ALGARVENSE*

Rodrigues MJ, Soszynski A, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L (2015). Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte *Limonium algarvense*. Industrial Crops and Products, 77, 315-322.

Rodrigues MJ, Neves V, Martins A, Rauter AP, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L (2016). *In vitro* antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: a comparison with green tea (*Camellia sinensis*). Food Chemistry, 200, 322-329.

Rodrigues MJ, Oliveira M, Neves V, Ovelheiro A, Pereira C, Neng NR, Nogueira JMF, Varela J, Barreira L, Custódio L (2019). Coupling sea lavender (*Limonium algarvense* Erben) and green tea (*Camellia sinensis* (L.) Kuntze) to produce an innovative herbal beverage with enhanced enzymatic inhibitory properties. South African Journal of Botany, 120, 87-94.

Rodrigues MJ, Monteiro I, Castañeda-Loaiza V, Placines C, Oliveira MC, Caperta AD, Pousão-Ferreira P, Pereira C, Custódio L. Growth performance, *in vitro* antioxidant properties and chemical composition of the halophyte *Limonium algarvense* Erben are strongly influenced by the irrigation salinity. Submitted for publication.

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

Maria João Rodrigues[a], Ambre Soszynski[a], Alice Martins[b], Amélia P. Rauter[b], Nuno R. Neng[b], José M.F. Nogueira[b], João Varela[a], Luísa Barreira[a], Luísa Custódio[a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] Faculty of Sciences of the University of Lisbon, Centre of Chemistry and Biochemistry/Department of Chemistry and Biochemistry, Building C8, Floor 5, Campo Grande, 1749-016 Lisbon, Portugal

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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-

* Corresponding author. Fax.: +351 289800 051.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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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

1 mg/mL. Results are expressed as percentage of inhibition, relative to the positive control, and as IC_{50} 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 $CuSO_4$•$5H_20$ (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 IC_{50} 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 Fe^{2+} 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 dH_20 and 30 μL of a $FeCl_2$ 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 IC_{50} 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 Velioglu 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 ($AlCl_3$) 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 $AlCl_3$ (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 Boulanouar et al., (2013), modified to 96-well plates. Briefly, 50 μL of 2% $AlCl_3$-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 "Mediterranea 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 IC_{50} 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 IC_{50} 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 (Badarinath 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 IC_{50} of 0.09 mg/mL, similar to that of the standard used, BHT (IC_{50} = 0.11 mg/mL); roots, peduncles and leaves had also high RSA (Table 1). The extracts were less active against the $ABTS^{+\bullet}$ radical, but similarly to DPPH assay the best result was also observed with flowers (IC_{50} = 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 IC_{50} value of 0.29 mg/mL, and none of the samples was able to scavenge the NO radical and to 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, supressing 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*.

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.

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 (Naczk 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]	RTi (min)[b]	Compound[c]	Flowers	Peduncles	Leaves	Roots
1	1.5	Gallic acid	3.37	3.87	1.40	2.34
2	4.7	*p*-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. monopetalum* and *T. gallica* (Falleh 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

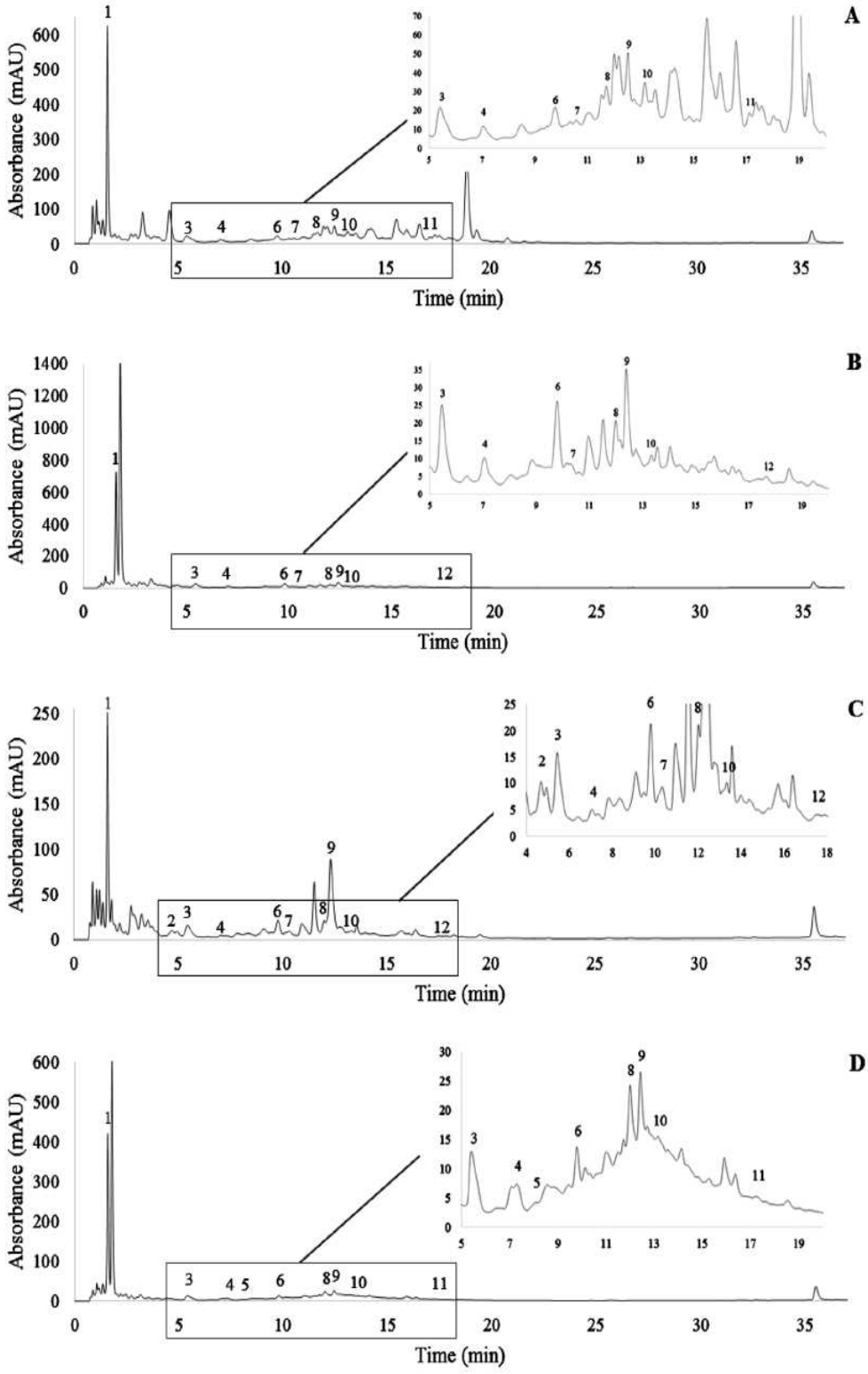

Fig. 1. HPLC–DAD analysis (280 nm) of phenolic compounds in methanol extracts of flowers (A), peduncles (B), leaves (C) and roots (D) of *L. algarvense*. The marked peaks refer to the compounds listed in Table 3.

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-

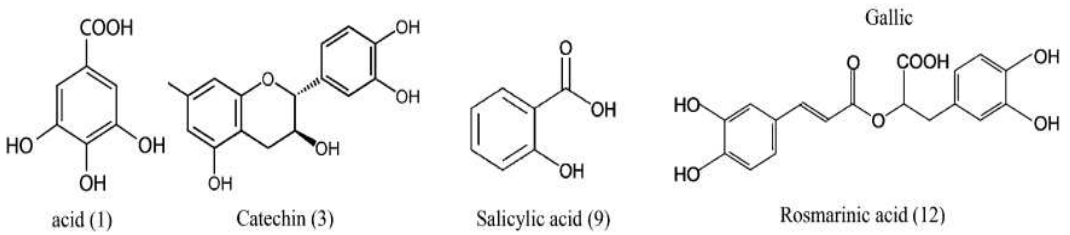

Fig. 2. Structures of some phenolic compounds detected in the methanolic extracts of flowers, peduncles, leaves and roots of *L. algarvense*. The numbers refer to the compounds listed in Table 3.

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. gemelinni* (roots) and *L. delicatulum* (shoots) (Anyia et al., 2002; Korulkina et al., 2004; Medini et al., 2014 Korulkina 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. gemelinni* roots (Korulkina 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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In vitro antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: A comparison with green tea (*Camellia sinensis*)

Maria João Rodrigues [a], Vanessa Neves [a], Alice Martins [b], Amélia P. Rauter [b], Nuno R. Neng [b], José M.F. Nogueira [b], João Varela [a], Luísa Barreira [a], Luísa Custódio [a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Ed. C8, 5º piso, 1749-016 Lisboa, Portugal

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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.

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

* Corresponding author.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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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, bronchorrhea, 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, cafeic, syringic, coumaric, salicylic, ferulic, rosmarinic, chlorogenic, gentisic, oleanolic, transcinnamic 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 transcinnamic 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 (IC_{50} values, mg/mL).

2.7. RSA on hydroxyl radical ($OH^{\bullet}$)

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 (H_2O_2, 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 IC_{50} 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 μL of Tris–HCl buffer (16 mM; pH 8.0), 50 μL of nitroblue tetrazolium (0.3 mM in Tri-HCl buffer), 50 μL of nicotinamide adenine dinucleotide solution (0.936 mM in a solution of sodium hydroxide 5 mM), 100 μL of the sample (at concentrations between 0.06 and 1 mg/mL) and 50 μL 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 IC_{50} values (mg/mL).

2.9. Ferric reducing antioxidant power (FRAP)

The ability of the extracts to reduce Fe^{3+} 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 IC_{50} 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 IC_{50} 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)/streptomicin (50 μg/mL), and incubated at 37 °C in humidified atmosphere with 5% CO_2.

2.12. Anti-inflammatory activity

The cell viability of the extracts (applied at concentrations ranging from 3 to 100 μg/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 μM) of sodium nitrite as standard. Results were expressed as a percentage relative to a control containing culture medium alone, and as IC_{50} values (μg/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 where 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 μg/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 μg/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 ± 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 IC_{50} 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
L. algarvense	Infusion	179 ± 2^c	96.0 ± 3.4^c	56.1 ± 7.4^b
	Decoction	191 ± 1^c	106 ± 5^c	44.0 ± 6.1^b
C. sinensis	Infusion	242 ± 3^b	150 ± 2^b	101 ± 16^a
	Decoction	290 ± 5^a	180 ± 5^a	145 ± 13^a

Values represent the mean ± 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

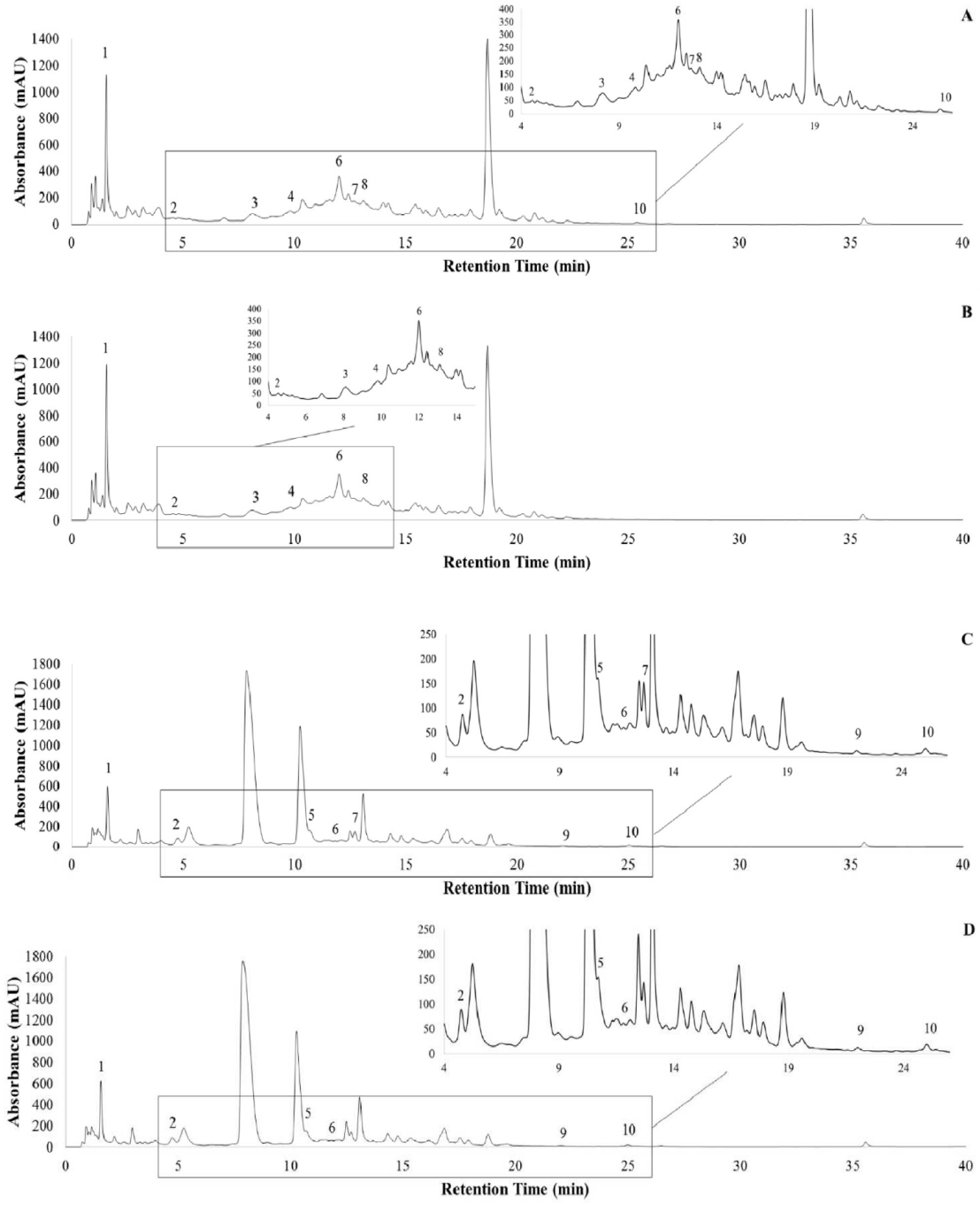

Fig. 1. HPLC–DAD analysis (280 nm) of phenolic compounds in infusions and decoctions of *L. algarvense* (A and B, respectively) and *C. sinensis* (C and D, respectively). Peak numbers refer to the compounds listed in Table 2.

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	*L. algarvense*		*C. sinensis*	
			Infusion	Decoction	Infusion	Decoction
1	1.58	Gallic acid	5.85	6.93	4.3	4.51
2	4.72	*p*-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-Grześkowiak, 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 ($OH^{\cdot}$) and superoxide ($O_2^{\cdot-}$) 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 IC_{50} values (mg/mL).

Species/compounds	Extract	DPPH$^{\cdot}$	OH$^{\cdot}$	$O_2^{\cdot-}$	CCA	ICA	FRAP
L. algarvense	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}
C. sinensis	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 showed any iron reducing activity at concentrations up to 1 mg/mL (Medini et al., 2014).

Superoxide ($O_2^{\bullet-}$) 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, $OH^{\cdot}$ are produced by the reaction of H_2O_2 and/or $O_2^{\cdot-}$ 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 $OH^{\cdot}$ (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 $O_2^{\cdot-}$, 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 addictive 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 $OH^{\cdot}$ 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 indicates 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 μg/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 pre-incubation 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 μM to around 16 μM (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 μg/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 μg/mL; decoction: 75.9 μg/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; Pragasam, 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 (μg/mL).

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

* Positive control. Values represent the mean ± 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 ($p < 0.05$).

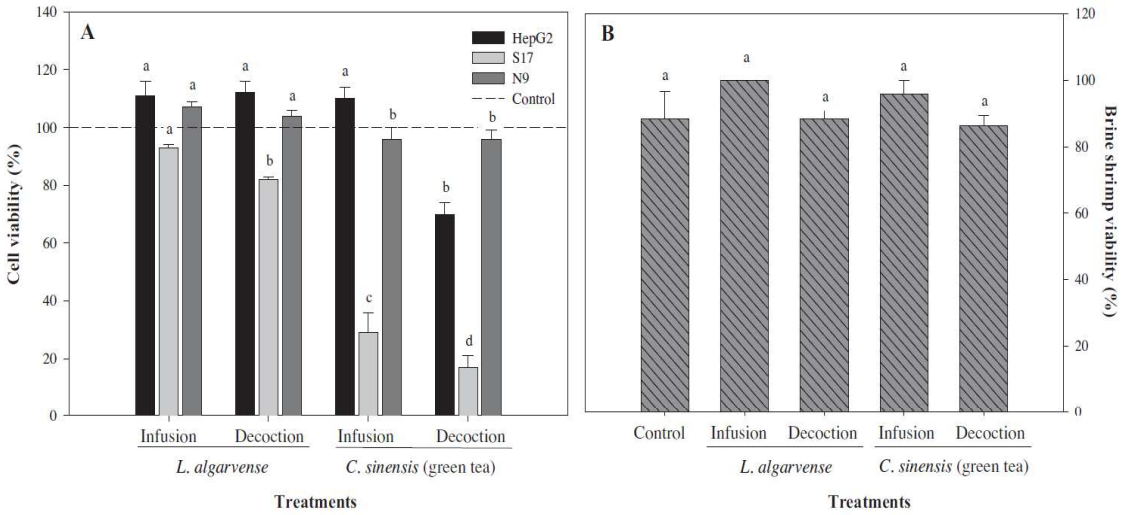

Fig. 2. Toxicity of the infusions and decoctions of *L. algarvense* and green tea on mammalian cell lines (HepG2, S17 and N9; A), and brine shrimp (B). Cells only treated with cell culture medium (A), and brine shrimps incubated only with artificial sea water (B) were used as controls. Values represent the mean ± 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 μg/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 μg/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. algarvense* 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. algarvense*. 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. algarvense* samples had similar radical scavenging activity against DPPH, hydroxyl and superoxide radicals, and also copper chelating activity than those of green tea. Nevertheless, *L. algarvense* 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. algarvense* extracts are non-toxic. Altogether, data obtained in this work strongly suggest that infusion and decoction of the halophyte *L. algarvense* 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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Coupling sea lavender (*Limonium algarvense* Erben) and green tea (*Camellia sinensis* (L.) Kuntze) to produce an innovative herbal beverage with enhanced enzymatic inhibitory properties

M.J. Rodrigues [a], M. Oliveira [a], V. Neves [a], A. Ovelheiro [a], C.A. Pereira [a], N.R. Neng [b], J.M.F. Nogueira [b], J. Varela [a], L. Barreira [a], L. Custódio [a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Ed. C8, 5° piso, 1749-016 Lisboa, Portugal

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ABSTRACT

Herbal beverages containing mixtures of *L. algarvense* (LA) flowers and *C. sinensis* (CS) were prepared and their inhibitory properties towards enzymes related with Alzheimer's (acetyl- and butyrylcholinesterase) and Type-2 *diabetes mellitus* (α-amylase and α-glucosidase) were evaluated for the first time. Samples were also appraised for antioxidant capacity and phenolic contents by high-performance liquid chromatography with diode-array detection (HPLC-DAD). Both synergistic and antagonistic interactions were observed: LA samples (infusion: IC_{50} = 0.22 mg/mL; decoction: 0.39 mg/mL) had higher acetylcholinesterase inhibition than CS and mixtures resulted in stronger enzymatic inhibition. CS had the highest rat α-glucosidase inhibition (infusion: IC_{50} = 3.91 mg/mL; decoction: IC_{50} = 2.50 mg/mL), which decreased when combined with LA. LA and CS samples had strong antioxidant activity, whereas LA and CS mixtures exhibited higher OH• radical-scavenging (0.56 ≤ SE ≤ 1.07) and anti-lipid peroxidation capacity (0.39 ≤ SE ≤ 0.81). CS had higher phenolic contents (infusion: 209 mg/g DW; decoction: 169 mg/g DW) and its combination with LA increased the phenolic diversity of the mixtures. Our results indicate that LA and CS infusions and decoctions and their combinations have relevant in vitro neuroprotective, antidiabetic and antioxidant properties and could be further explored as potential innovative functional beverages able to reduce the progression of neurodegenerative diseases and diabetic complications, and to prevent oxidative stress and lipid oxidation related diseases.

1. Introduction

Studies aiming the valorisation of medicinal plants as sources of innovative natural products and/or molecules for both traditional and modern medicine are re-emerging (Qureshi et al., 2016). In particular, reports of the in vitro inhibitory properties of natural products on enzymes related with human health diseases (e.g. Alzheimer's and diabetes) are particularly relevant. This strategy is being extensively embraced by different researchers for several plant species, as for example *Stachys annua* L., *Lycium barbarum* L., *Schisandra chinensis* (Turcz.) Baill and *Euphorbia denticulata* Lam (Mocan et al., 2016, 2017; Kocak et al., 2017; Zengin et al., 2017).

Herbal beverages such as teas (made from *Camellia sinensis* (L.) Kuntze leaves) and tisanes (made from herbs, spices, or other plant parts) have been used for centuries in traditional medicine for the treatment of several human diseases, including neurological disorders and hyperglycaemia (Atoui et al., 2005). Particularly, green tea which results from the fast drying of the fresh leaves of *C. sinensis* has several confirmed health promoting properties comprising antioxidant, anti-inflammatory, anti-diabetic and neuroprotective (Uysal et al., 2013). In addition, different *Limonium* species are used in Chinese, European, Latin America and Arabian traditional medicines for the treatment of different health problems, as for example cardiovascular and inflammatory problems (Murray et al., 2004). *Limonium algarvense* Erben (sea lavender, Fig. 1) is an obligatory halophyte plant endemic to the Southwest area of the Iberian Peninsula (Tutin et al., 1972). Infusions and decoctions of this species, especially from the flowers, have high in vitro antioxidant and anti-inflammatory activities, similar to green tea, and are rich in phenolic compounds, mainly salicylic, gallic and coumaric acids (Rodrigues et al., 2016).

Alzheimer's disease (AD) accounts for approximately 60–70% of dementia cases and affects around 44 million people nowadays, a number that is estimated to increase to 66 million by 2030 and to 115 million by 2050 (ADI, 2016). This disease has no cure and is a major cause of death in the elderly (Kumar et al., 2015). AD hallmarks include the death of neurons located in the hippocampus region of the brain, which is responsible for memory and learning, and also by beta-amyloid peptide

* Corresponding author.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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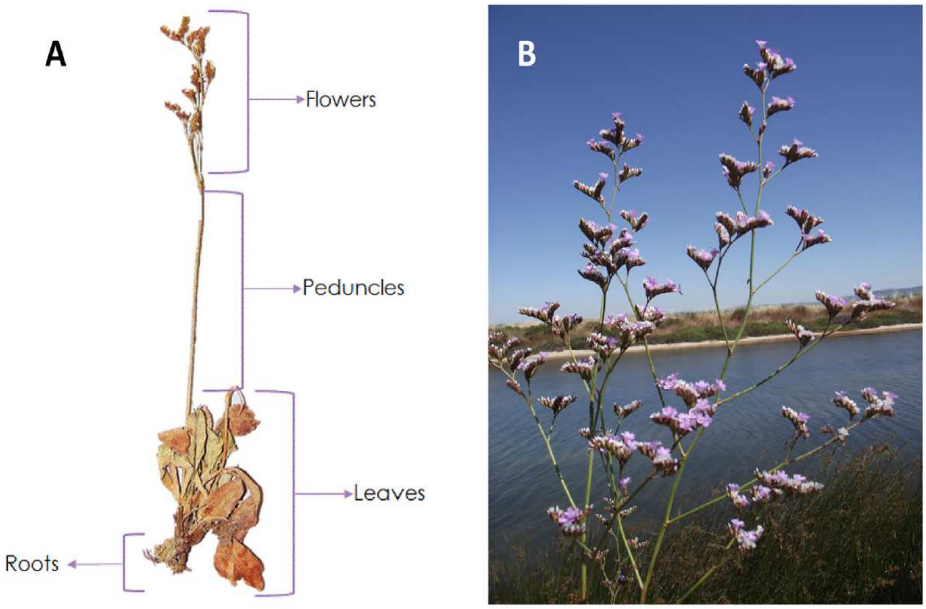

Fig. 1. Schematic representation of a *Limonium algarvense* plant (A) and detail of the flowers (B).

and tau protein accumulation in this area (Kumar et al., 2015). AD is also characterized by reduced levels of the neurotransmitter acetylcholine (ACh), which is hydrolysed by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The inhibition of the latter enzymes can raise the ACh levels in the synaptic cleft, thus improving cognitive functions (Ballard, 2002).

Diabetes mellitus affected approximately 422 million people worldwide in 2014, and it is projected to be the 7th leading cause of death in 2030 (WHO, 2016). Type 2 diabetes (T2DM) represents more than 90% of all diabetes cases, and results mainly from genetics and lifestyle features (Mozaffarian et al., 2009). T2DM is characterized by the occurrence of high blood glucose levels (hyperglycaemia), which results from insulin secretion modifications or low receptiveness to the secreted insulin (Yarchoan and Arnold, 2014). One of the most recognized therapeutic approaches for the control of T2DM is the use of inhibitors of carbohydrate-hydrolysing enzymes, such as α-amylase and α-glucosidase, since they reduce the breaking down of starch into sugar monomers thus lowering the postprandial blood glucose levels (Kwon et al., 2007). Moreover, recent studies indicate that patients with T2DM have higher risk of developing AD, which suggests a link between those diseases (de la Monte and Wands, 2008; Yarchoan and Arnold, 2014). Although the exact nature of the association between AD and diabetes is not yet fully understood, it may be related for example, with an increased risk of developing heart diseases, which can damage the brain blood vessels, unbalanced brain signaling due to high concentrations of insulin and also with hyperglycaemia-induced inflammation that may damage brain cells (Li and Hölscher, 2007). AD is thus indicated as a brain-specific form of diabetes called "type 3 diabetes", due to the shared molecular and biochemical features typical of diabetes, including expression of genes encoding for insulin, insulin growth factors, receptors and related signaling molecules (Li and Hölscher, 2007; de la Monte and Wands, 2008). The link between these diseases may also include inflammation, oxidative stress, amyloid and tau protein depositions and inadequate ACh production (Yarchoan and Arnold, 2014). In this sense, it is currently accepted that AD patients might benefit from therapies used for the treatment of T2DM (Akter et al., 2011). In fact AD patients treated with anti-diabetic medications have shown cognitive improvement or stabilization (Li and Hölscher, 2007; de la Monte and Wands, 2008).

Natural products (including herbal beverages) with the capacity to prevent and/or manage chronic diseases, like AD and diabetes, are a promising way to continuously improve human well-being and relieve social systems through health promotion. Moreover, folk medicine frequently combines different plant species in mixtures, called herbal mixtures, with the purpose to increase the individual pharmacological properties and/or to reduce their toxicity (Kuijun et al., 2009). For example, green tea is commonly sold in mixtures combining different herbs, fruits or spices (e.g. lemon, ginger, mint, anise, cinnamon, jasmine, raspberry, pomegranate) in order to improve or add beneficial properties to such beverages (Jain et al., 2011). Having this in mind, and following previous promising results on the antioxidant and anti-inflammatory activities of sea lavender flowers' infusions and decoctions, this work prepared and evaluated innovative herbal beverages containing mixtures of sea lavender and green tea in terms of inhibitory activities on enzymes related with Alzheimer's and T2DM and also antioxidant capacity. The phenolic profile of these beverages was also determined by high-performance liquid chromatography with diode-array detection (HPLC-DAD).

2. Experimental

2.1. Chemicals

Acetylcholinesterase (AChE, EC 3.1.1.7), butyrylcholinesterase (BChE, EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine iodide, 5-thio-2-nitrobenzoate (DTNB), α-amylase from porcine pancreas (EC 3.2.1.1), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), intestinal acetone powders from rat, butylated hydroxytoluene (BHT) and catechin were purchased from Sigma-Aldrich.

2.2. Plant material

Samples from *L. algarvense* (Fig. 1) were collected in the South of Portugal (Ludo) in June of 2015 (coordinates: 37°2′6.526″N 7°58′ 58.465″W). The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) and a voucher specimen is kept in the herbarium of the MarBiotech laboratory (voucher code MBH01). The flowers were dried for 3 days at 40 °C, powdered and stored at −20 °C until needed. Green tea was bought in a regional supermarket, powdered and also stored at −20 °C. For the analysis of mixtures, the dried powder of *L. algarvense* flowers and green tea

were mixed in different proportions, namely 3:1, 1:1 and 1:3, before extraction.

2.3. Extraction

Infusions were prepared by mixing 1 g of dried samples with 200 mL of ultrapure boiling water for 5 min. Decoctions were prepared by boiling 1 g of dried sample in 200 mL of ultrapure water for 5 min. Extracts were filtered (Whatman no. 4), freeze dried and dissolved in ultrapure water. Three independent extractions ($n = 3$) of the dried samples were made and extracts resulting from the different extractions were tested for their bioactivities. As no significant differences were found among corresponding extracts from the different extractions, for the HPLC analysis the extracts were freeze-dried and pooled accordingly and stored at −20 °C protected from the light.

2.4. Identification and quantification of phenolic compounds by HPLC-DAD

The extracts at the concentration of 10 mg/mL in ultrapure water were analyzed by HPLC-DAD (Agilent 1100 Series LC system, Germany), as described before (Rodrigues et al., 2016). The identification of the compounds was based on their retention times comparing with those of commercial standards. Peak purity was assessed using the UV–visible spectra of reference data. Levels of the different compounds were determined using calibration standard curves made for each of the commercial standards (catechol, catechin hydrate, epigallocatechin gallate, 4-hydroxybenzaldehyde, epicatechin, and gallic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, salicylic, ferulic, chlorogenic, neochlorogenic and gentisic acids) prepared in methanol (10,000 mg/L) and diluted with ultrapure water to the desired concentration.

2.5. In vitro inhibition of enzymes related with neurological disorders

2.5.1. AChE and BChE inhibitory activities

Samples (0.1–10 mg/mL) were evaluated for their capacity to inhibit AChE and BChE activities by the Ellman's method as described in Custódio et al. (2015). Galantamine (0.01–1 mg/mL) was used as the standard, and results were expressed as inhibition (%) relatively to a control containing water in place of the sample, and as IC_{50} values (mg/mL), for those extracts exhibiting inhibition higher than 50% when tested at 10 mg/mL.

2.6. In vitro inhibition of enzymes related with type 2 diabetes

2.6.1.1. Alpha-amylase inhibitory activity. Inhibition of α-amylase was determined by the method described by Xiao et al. (2006) on extracts at concentrations ranging from 1 to 10 mg/mL. Acarbose was used as the positive control at the same concentrations of the extracts, and results were expressed as inhibition (%) relatively to a blank containing water, and when possible as IC_{50} values (mg/mL).

2.6.1.2. Baker's yeast α-glucosidase inhibitory activity. The Baker's yeast α-glucosidase inhibitory activity was determined according to the method described by Kwon et al. (2007). Samples were tested at different concentrations (0.01 to 1 mg/mL) and results were expressed as inhibition (%) related to a control containing water, and as IC_{50} values (mg/mL). Acarbose was used as the standard at the same concentrations used for the samples.

2.6.1.3. Rat's intestinal α-glucosidase inhibitory activity. In this assay rat's intestinal acetone powder was used as a crude enzyme extract as an example of enzyme of mammalian origin (Kwon et al., 2007). The extracts were tested at different concentrations (1 to 10 mg/mL) and results were expressed as inhibition (%) related to a control containing water, and whenever possible, as IC_{50} values (mg/mL). Acarbose was used as positive control at the same concentrations of the extracts.

2.7. Antioxidant activity

2.7.1. Radical scavenging activity (RSA) on hydroxyl (OH•) and superoxide ($O_2^{\bullet-}$) radicals

The hydroxyl and superoxide RSA were tested on extracts at concentrations ranging from 0.1 to 10 mg/mL, by the methods described in Rodrigues et al. (2016). Catechin was used as standard at the same concentrations of the samples, and results were expressed as percentage of inhibition, relative to a negative control containing ultrapure water in place of the sample, and as half maximal inhibitory concentration (IC_{50} values, mg/mL).

2.7.2. Ferric thiocyanate (FTC) test

The inhibition of linoleic acid peroxidation was measured according to the method described by Bouaziz et al. (2015) with some modifications. Samples (0.5 mL) at different concentrations (0.1 to 10 mg/mL) were incubated with 2 mL of 0.2 M phosphate buffer (pH 7) and 2.5 mL of 0.02 M linoleic acid emulsion (0.28 g of linoleic acid and 0.28 g of Tween-20 in 50 mL of the previous buffer). After an incubation period of 72 h at 40 °C, samples were mixed with 5 mL of 75% ethanol (0.1 mL), 30% ammonium thiocyanate (0.1 mL) and 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.1 mL). Three minutes after, the absorbance was measured at 500 nm. The measurement was performed each 24 h until the absorbance of the control achieved the maximum value. Butylated hydroxytoluene (BHT) was used as the reference at the same concentrations of the samples, and results were expressed as percentage of inhibition, relatively to a control containing ultrapure water in place of the sample, and as IC_{50} values (mg/mL), for those extracts exhibiting RSA higher than 50% when tested at 10 mg/mL.

2.7.3. Thiobarbituric acid (TBA) test

The TBA test was conducted on the final day of FTC test as described before (Bouaziz et al., 2015). Samples were prepared as described for the FTC test; after the incubation time, 1 mL of the samples was mixed with 20% trichloroacetic acid (2 mL) and 0.67% thiobarbituric acid (2 mL). This mixture was incubated in a boiling water bath for 10 min and left to cool. After centrifugation at 3000 rpm for 20 min, the absorbance of the supernatants was read at 532 nm. BHT was used as a standard at the same concentrations of the samples, and results were expressed as percentage of inhibition, relative to a blank containing ultrapure water, and as IC_{50} values (mg/mL), for those extracts exhibiting inhibitions higher than 50% when tested at 10 mg/mL.

2.8. Determination of synergistic effects (SE) of herbal mixtures

In order to make a comparative evaluation of the biological activities of the individual and combined infusions and decoctions, the synergistic effect was calculated using the following equation: SE = Experimental value (EV) / Theoretical value (TV). TV was determined through the weighted average of individual extracts' activity for each of the combined extracts, whereas EV refers to the values obtained in the experiments (Nedamani et al., 2015). When IC_{50} values were higher than 10 mg/mL, the SE was calculated using this concentration, indicating the maximum SE value that can be obtained. The observed effect is considered as synergistic when $SE < 1$, additive if $SE = 1$ and antagonistic when $SE > 1$.

2.9. Statistical analysis

Results were expressed as mean ± 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 the Duncan's test, and by the Kruskal-Wallis test when

parametricity did not prevail. Differences were considered significant when p values were lower than 0.05. All statistical analyses were performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft corporation). The IC_{50} values were calculated by sigmoidal fitting of the data using the GraphPad Prism v. 5.0 program.

3. Results and discussion

3.1. Phytochemical analysis

The phytochemical composition of infusions and decoctions of sea lavender flowers (*L. algarvense*, LA), green tea (*C. sinensis*, CS), and their mixtures were assessed through the identification and quantification of phenolic compounds by HPLC-DAD analysis, and results are summarized on Table 1 and Fig. S1.

From the 16 standards tested, 13 were detected in LA and CS infusions and decoctions, and in their combinations. The sum of the phenolics detected in CS was higher (infusion: 209 mg/g DW; decoction: 169 mg/g DW), than in LA (infusion: 17.1 mg/g DW; decoction: 17.3 mg/g DW), and as the CS proportion increases in the mixtures the total sum of phenolics also increases, in a ratio-dependent manner. The main compounds found in LA infusion and decoctions were salicylic (infusion: 9.03 mg/g DW; decoction: 8.86 mg/g DW) and gentisic (infusion: 3.88 mg/g DW; decoction: 3.65 mg/g DW) acids, whilst epicatechin and caffeic acid were mainly detected in CS infusions (140 and 49.4 mg/g DW, respectively) and decoctions (104 and 51.0 mg/g DW).

Some particular compounds were identified only in LA or CS, as for example *p*-hydroxybenzoic, caffeic and syringic acids that were only detected in CS infusions, whereas vanillic acid was solely identified in LA infusions. However, those compounds were also present in the different mixtures of LA and CS in a ratio-dependent manner. Coumaric acid was identified in LA alone (infusion and decoction), but not in CS or in their mixtures, probably due to its low concentration. Concerning the decoctions, a similar pattern than in infusions was observed with *p*-hydroxybenzoic, caffeic and vanillic acids. Conversely, syringic acid was only detected in CS decoction but not in the mixtures with LA since its amount decreased to below the detection limit as the LA ratio increases in the mixture. The phenolic compounds identified in both LA and CS infusions and decoctions, as well as in LA + CS mixtures showed a concentration dependent on the used ratio, that is, the detected levels increased/decreased as the proportion of LA or CS increases/decreases. This indicates that the compounds' concentration in the mixtures reflects the proportions of the initial solutions, indicating that there was no degradation or formation of new compounds.

Catechins are flavonoids typically present in *C. sinensis*, namely epicatechin that was found in high amounts in CS infusion and decoction. This finding sustains the use of green tea as a rich dietary source of these molecules which are known to have extensive benefits for human health, including antioxidant, antidiabetic and neuroprotective properties (Rains et al., 2011; Vallverdú-Queralt et al., 2015). Besides, the values obtained in this work are in accordance with those found in the literature, that indicate that a cup of green tea should contain 50–100 mg of catechins (Rains et al., 2011). In turn, LA extracts had higher contents of hydroxybenzoic acids, such as salicylic and gentisic acids, which are frequently found in most of dietary herbs, as for example caraway, turmeric, dill and marjoram, conferring them health-promoting properties, such as antimicrobial, digestive stimulant, anti-inflammatory, antioxidant and anticarcinogenic activities (Vallverdú-Queralt et al., 2015). Thus, combining LA with CS enhances the beneficial properties of these beverages, by increasing the chemical diversity.

Gallic, ferulic, vanillic and coumaric acids were previously identified in polar extracts (methanol and water) of different *Limonium* species, as well as salicylic acid that was already detected in *L. algarvense* from the same region (Rodrigues et al., 2016). To the best of our knowledge this is the first report of the occurrence of catechol, epicatechin, gentisic and neochlorogenic acids in the *Limonium* genus. Although from the same region, the plants were collected in different years, which may lead to the detection of different compounds (epicatechin and gentisic acid) that may be present in the extracts at undetectable levels on the previous study (Rodrigues et al., 2016). Regarding green tea, epicatechin, gallic, caffeic, ferulic and salicylic acids were formerly reported in aqueous extracts of *C. sinensis* (Rodrigues et al., 2016). Although the phenolics of green tea are already well characterized, we could not find any previous study describing the detection of catechol, neochlorogenic, gentisic and syringic acids in *C. sinensis*.

3.2. In vitro inhibition of enzymes related with neurological disorders

Infusions and decoctions of LA had a strong capacity to inhibit AChE, with IC_{50} values of 0.22 and 0.39 mg/mL, respectively (Table 2). Although less effective than the standard used in the assay (galantamine: IC_{50} = 0.01 mg/mL), LA samples were significantly more efficient than CS (Table 2) against AChE. Samples had a lower inhibitory capacity towards BChE, but again, LA allowed better results than CS, which was ineffective towards this enzyme (Table 2). Herbal mixtures containing LA and CS had at least 6-times lower IC_{50} values on AChE when compared with those obtained with CS alone, which points to a strong

Table 1
High performance liquid chromatography (HPLC-DAD) analysis of phenolic compounds content (mg/g DW) of infusions and decoctions of sea lavender (*L. algarvense*, LA), green tea (*C. sinensis*, CS), and their mixtures.

Peak no.[a]	RT (min)[b]	Compound	Infusions					Decoctions				
			LA	LA + CS 3:1	LA + CS 1:1	LA + CS 1:3	CS	LA	LA + CS 3:1	LA + CS 1:1	LA + CS 1:3	CS
1	1.5	Gallic acid	1.47	1.96	2.38	2.78	3.38	1.84	2.53	2.71	3.54	3.39
2	2.5	Catechol	0.62	0.60	0.39	0.20	0.11	0.69	0.56	0.39	0.16	0.13
3	2.8	Neochlorogenic acid	0.29	1.01	1.34	1.94	2.58	–	–	1.02	2.74	2.56
4	4.1	Gentisic acid	3.88	2.70	1.66	1.04	3.30	3.65	4.64	3.16	3.96	1.96
5	4.4	*p*-Hydroxybenzoic acid	–	0.09	0.39	0.88	1.74	–	0.13	0.51	0.96	1.65
6	5.1	Catechin hydrate	–	–	–	–	–	–	–	0.57	–	–
7	6.4	Vanillic acid	0.17	0.1	0.04	0.01		0.16	0.09	0.05	–	–
8	7.7	Caffeic acid	–	16.0	33.2	39.9	49.4	–	13.2	26.2	49.3	51.0
9	9.2	Syringic acid	–	–	0.05	0.05	0.03	–	–	–	–	0.02
10	10.5	Epicatechin	1.18	12.2	20.3	32.3	140	1.68	18.3	42.2	98.4	104
11	11.6	Coumaric acid	0.04	–	–	–	–	0.06	–	–	–	–
12	11.8	Salicylic acid	9.03	5.08	3.12	1.95	2.47	8.86	4.88	2.94	1.89	0.81
13	13	Ferulic acid	0.44	0.07	0.43	1.13	5.85	0.41	0.05	1.71	4.56	3.95
		Total	17.1	39.9	63.3	82.2	209	17.3	44.5	81.5	165	169

[a] Corresponding peak number in the chromatogram showed on Fig. S1 in Supplementary material.
[b] Retention times. –: not detected.

Table 2
Inhibitory activity (IC_{50} mg/mL) and synergistic effects (SE) of infusions and decoctions of sea lavender (*L. algarvense*, LA), green tea (*C. sinensis*, CS), and their mixtures on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Results are expressed as IC_{50} values (mg/mL).

Sample/compound		AChE		BChE	
		IC_{50}	SE	IC_{50}	SE
Infusions	LA	0.22 ± 0.01^{b}	–	0.84 ± 0.04^{b}	–
	LA + CS 3:1	0.64 ± 0.00^{cd}	0.65	0.28 ± 0.01^{a}	<0.18
	LA + CS 1:1	0.48 ± 0.01^{bc}	0.69	0.25 ± 0.02^{a}	<0.19
	LA + CS 1:3	0.92 ± 0.06^{de}	0.26	1.40 ± 0.02^{e}	<0.09
	CS	7.24 ± 0.31^{g}	–	>10	–
Decoctions	LA	0.39 ± 0.02^{bc}	–	0.96 ± 0.01^{c}	–
	LA + CS 3:1	0.64 ± 0.02^{cd}	0.21	0.31 ± 0.03^{a}	<0.11
	LA + CS 1:1	0.34 ± 0.02^{bc}	0.34	0.31 ± 0.01^{a}	<0.36
	LA + CS 1:3	0.98 ± 0.07^{e}	0.42	1.28 ± 0.11^{d}	<0.33
	CS	6.17 ± 0.0^{f}	–	>10	–
Galantamine*		0.01 ± 0.00^{a}		0.32 ± 0.01^{a}	

* Positive control. SE: Synergistic effect (SE < 1: synergistic; SE > 1: antagonistic). 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 test ($p < 0.05$).

synergistic effect (SE < 1, Table 2). The application of infusions and decoctions of LA and CS at 3:1 and 1:1 ratios improved the enzymatic inhibition of BChE, also indicating a synergistic effect (SE < 1, Table 2).

There is a growing consumer's trend to use natural products, including those aiming to improve cognitive functions, and different herbal products from different plant species (e.g. *Hypericum perforatum* L., *Lavandula officinalis* Mill., *Ginkgo biloba* L., *Salvia officinalis* L., *Melissa officinalis* L., *Panax ginseng* C. A. Mey.) have been developed for the improvement of cognitive functions in patients with mild-to-moderate AD, by cholinesterase inhibition and oxidative stress reduction (Jivad and Rabiei, 2014). Thus, plants used in traditional medicine are being used as sources of novel products to be used as functional foods or beverages (Jivad and Rabiei, 2014). Our results suggest that infusions and decoctions of LA and CS at 3:1 and 1:1 ratios can be used as innovative functional beverages for this purpose.

Phenolic compounds are efficient inhibitors of AChE and BChE, particularly some phenolic acids present in LA, such as gallic, vanillic, caffeic, gentisic and salicylic acids (Szwajgier, 2015), which may explain the significant in vitro AChE and BChE inhibition of infusion and decoction of LA and its combinations with CS. However, and in contrast to the findings of Szwajgier (2015), the combination of different phenolic compounds did not decrease the anti-cholinesterase activity, but rather increased it synergistically (SE < 1). This may be due to the presence of other phenolics in our samples that originate different regeneration mechanisms which contribute to the increased activity, as discussed above for antioxidant activity (Peyrat-Maillard et al., 2003). Moreover, some cholinesterase inhibitors, as for example pyridostigmine, have the capacity to improve bowel motility in diabetic patients suffering of constipation, which is one of the main issues of diabetics (Bharucha et al., 2013). The cholinesterase inhibitor neostigmine was also able to modulate inadequate bladder contractions, usually seen in later stage diabetic patients (Mustafa and Ismael, 2014). For these reasons, infusions and decoctions of LA + CS may hold the potential to be used as functional neuroprotective herbal beverages, as well as in diabetic complications related with increased cholinesterase activity.

There are several studies confirming that green tea consumption is inversely correlated with the loss of cognitive skills and with the incidence of neurodegeneration, namely AD (Kuriyama et al., 2006). The neuroprotective properties of green tea are mainly attributed to its phenolic contents, particularly the levels of catechins which act by different mechanisms, as for example, antioxidant features, modulation of various protein kinase signaling pathways and AChE inhibition (Okello et al., 2012). The AChE inhibition of green tea observed in this study was lower than previously reported values (Okello et al., 2012), possibly due to differences in the methodology used for the evaluation of the inhibitory capacity and also to different origins of the plants. Regarding the AChE inhibitory potential of species belonging to the *Limonium* genus, in a previous report, a chloroform extract of *L. okotranum* had moderate activity on AChE (Dhivya et al., 2014). To the best of our knowledge there were no reports until now about the potential neuroprotective properties of *L. algarvense* in general, and of its cholinesterase inhibitory capacity, in particular.

3.3. In vitro inhibition of enzymes related with T2DM

As can be seen on Table 3, samples were generally more active against α-glucosidases that on α-amylase, which none of the samples was able to inhibit even at the highest concentration tested (10 mg/mL). This agrees with previous reports on the higher inhibitory activity against α-glucosidase of natural compounds from plants, comparatively with α-amylase (Kwon et al., 2007). Moreover, and similar to what is generally reported in the literature, samples had a stronger inhibitory capacity towards microbial α-glucosidase than to α-glucosidase from mammalian origin (Shai et al., 2011).

Similar to the results obtained with the inhibitory capacity on AChE and BChE, LA had a higher inhibitory capacity towards enzymes related with diabetes. The highest activity against baker's yeast α-glucosidase was observed after the application of individual infusions and decoctions of LA, with IC_{50} values of 0.05 and 0.04 mg/mL, respectively (Table 3). Green tea also allowed for a significant reduction of microbial α-glucosidase activity, with similar IC_{50} values for infusions and decoctions (0.12 mg/mL, Table 3). The application of different ratios of LA and CS samples significantly reduced enzymatic inhibition, denoted by the higher IC_{50} values, thus suggesting an antagonistic effect (SE > 1, Table 3). Noteworthy is the fact that all the extracts were statistically

Table 3
Inhibitory activity (IC_{50} mg/mL) and synergistic effects (SE) of infusions and decoctions of sea lavender (*L. algarvense*, LA), green tea (*C. sinensis*, CS), and their mixtures on α-amylase, baker's yeast α-glucosidase and rat's intestinal α-glucosidase. Results are expressed as IC_{50} values (mg/mL).

Sample/compound		α-Amylase	Yeast α-glucosidase		Rat α-glucosidase	
		IC_{50}	IC_{50}	SE	IC_{50}	SE
Infusions	LA	>10	0.05 ± 0.00^{a}	–	>10	–
	LA + CS 3:1	>10	0.17 ± 0.01^{d}	5.02	>10	<2.36
	LA + CS 1:1	>10	0.14 ± 0.00^{c}	5.80	5.76 ± 0.65^{c}	<1.88
	LA + CS 1:3	>10	0.36 ± 0.01^{f}	3.24	5.11 ± 0.39^{bc}	<1.67
	CS	>10	0.12 ± 0.00^{bc}	–	3.91 ± 0.52^{b}	–
Decoctions	LA	>10	0.04 ± 0.00^{a}	–	>10	–
	LA + CS 3:1	>10	0.16 ± 0.00^{d}	6.56	7.62 ± 0.39^{d}	<2.29
	LA + CS 1:1	>10	0.25 ± 0.00^{e}	6.87	7.15 ± 0.30^{d}	<1.88
	LA + CS 1:3	>10	0.34 ± 0.02^{f}	7.07	5.12 ± 0.26^{bc}	<2.35
	CS	>10	0.12 ± 0.00^{b}	–	2.50 ± 0.20^{a}	–
Acarbose*		7.80 ± 0.10	3.14 ± 0.13^{g}		4.64 ± 0.44^{bc}	

* Positive control. SE: Synergistic effect (SE < 1: synergistic; SE > 1: antagonistic). 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 test ($p < 0.05$).

more active than the positive control (acarbose, $p < 0.05$). Regarding the mammalian α-glucosidase, the lowest IC_{50} values were obtained with individual CS decoctions, followed by its infusion, whereas no inhibition was obtained after the application of LA samples (Table 3). The inhibitory activity of the mixtures decreased with the increasing ratio of LA, which was not active when applied alone (Table 3). This antagonistic effect may also occur due to regeneration mechanisms of the OH groups among phenolic acids and flavonoids, where the more efficient molecule regenerates the less active one, leading to decreased activity of the mixtures (Peyrat-Maillard et al., 2003). However, the infusion and decoction of LA + CS at the proportion 1:3 allowed for IC_{50} values similar to those obtained with the standard (acarbose; $p < 0.05$).

The most frequently used medications for T2DM management include carbohydrate-hydrolysing enzymes, as for example α-glucosidase inhibitors, which reduces the breaking down of starch into sugar monomers, contributing to decrease the postprandial blood glucose levels (Kwon et al., 2007). Several dietary plant species are used in traditional medicine as source of bioactive phenolic compounds, which are particularly important to provide health benefits to foods and beverages, contributing to the management of oxidative stress-related diseases, such as hyperglycaemia linked to T2DM (Kwon et al., 2007). Actually, the major phenolic compounds detected in CS infusions and decoctions have been previously described as having carbohydrate digestive enzymes inhibitory properties. Catechins, particularly epicatechin that was detected in CS infusions and decoctions in a high concentration, showed strong α-glucosidase inhibition but was not active against α-amylase (Yilmazer-Musa et al., 2012). Caffeic acid was the second major compound identified in CS extracts and has also a strong α-glucosidase inhibitory capacity (Oboh et al., 2015). Thus, the presence of those compounds in green tea infusions and decoctions can be responsible for the high capacity to inhibit dietary carbohydrate digestive enzymes, contributing to the control of the glucose levels in diabetics. These beverages can also be used to decrease the risk of developing AD in T2DM patients, since hyperglycaemia is considered one of the major risk factors for developing AD (Li and Hölscher, 2007).

3.4. Antioxidant activity

Folk medicine frequently combines different plant species in mixtures (herbal mixtures), with the intent of increasing their individual pharmacological properties or to reduce their toxicity. In these mixtures, different types of interactions can be obtained, namely antagonistic (when the sum of the effects is lower than the predicted mathematical sum of the individual components), synergistic (when the effect is higher than the sum of individual components) or addictive effects (when the mixture provides the sum of the effects of the individual components) (Kuijun et al., 2009; Wang et al., 2011). In this context we targeted the identification of synergistic interactions between samples from LA and CS aiming the development of herbal mixtures with the potential to minimize diseases associated with oxidative stress. Having this in mind, the antioxidant capacity of infusions and decoctions of sea lavender and green tea, along with their mixtures, was evaluated by four different and complementary methods, including RSA on the OH• and O_2^{-}, and anti-lipid peroxidation activity (FTC and TBA methods). Results are depicted in Table 4.

Infusions and decoctions of LA and CS had a strong antioxidant activity, and both species had similar capacity to scavenge OH• and O_2^{-}•; however, LA was more efficient in the early stages of lipid oxidation by inhibiting peroxide production (FTC) and also in preventing the decomposition of peroxide in the final stages of lipid peroxidation (TBA). The capacity to scavenge the OH radical and to prevent lipid peroxidation (TBA) was significantly improved when mixtures of LA and CS were applied, comparatively to the application of the individual extracts, regardless of the proportion used. This result suggests a synergistic effect of the herbal combinations (SE < 1), as described before for other herbal mixtures with green tea, namely with rosemary (*Rosmarinus officinalis* L.) and oak fruit (*Quercus branti* Lindl.) (Nedamani et al., 2015), and also with common grape vine (*Vitis vinifera* L.), Indian gooseberry (*Phyllanthus emblica* L.), pomegranate (*Punica granatum* L.), Chinese cinnamon (*Cinnamomum cassia* (L.) J. Presl) and ginkgo (*Ginkgo biloba* L.) (Jain et al., 2011). Regarding the antiradical activity towards O_2^{-}• a clear antagonistic effect was obtained with the application of herbal mixtures (SE > 1). The SE of LA and CS combinations may be explained by regeneration mechanisms of the OH groups of phenolic acids and flavonoids, where a more efficient molecule can be regenerated by a less active one, as suggested by Peyrat-Maillard et al. (2003).

Several physiological and metabolic processes occurring in aerobic organisms, as for example the mitochondrial respiration, produce reactive oxygen species (ROS), such as O_2^{-}• and OH• (Koppenol, 2001). The radical OH is produced in the Haber-Weiss reaction through the reaction of H_2O_2 and/or O_2^{-}• with Fe^{3+}, and also in the Fenton reaction, where Fe^{2+} reacts with H_2O_2 (Koppenol, 2001). This oxidative state induces damages in important cellular macromolecules (e.g. DNA, proteins and lipids) and leads to the increased incidence of diverse pathological conditions, namely T2DM and neurodegenerative diseases (Uttara et al., 2009). Brain consumes around 20–30% of the inhaled oxygen and contains high contents of polyunsaturated fatty acids, making this organ highly susceptible to lipid peroxidation. This fact contributes to severe neuronal membranes damage and to brain dysfunction, which is currently associated with neurodegeneration and the development of AD and Parkinson's diseases (Reed, 2011). Antioxidants (e.g. vitamin C, vitamin E, glutathione) are known to prevent and/or counteract oxidation, namely through the scavenging

Table 4
Radical-scavenging activity on hydroxyl (OH•) and superoxide (O_2^{-}•) radicals, and inhibition of lipid peroxidation (ferric thyocianate; FTC and thiobarbituric acid: TBA tests) of infusions and decoctions of sea lavender (*L. algarvense*, LA), green tea (*C. sinensis*, CS), and their mixtures. Results are expressed as IC_{50} values (mg/mL) and as synergistic effects (SE).

Sample/compound		OH•		O_2^{-}•		FTC		TBA	
		IC_{50}	SE	IC_{50}	SE	IC_{50}	SE	IC_{50}	SE
Infusions	LA	1.13 ± 0.03^{e}	–	0.11 ± 0.01^{a}	–	6.37 ± 0.01^{b}	–	3.23 ± 0.06^{f}	–
	LA + CS 3:1	0.34 ± 0.02^{bc}	0.64	0.37 ± 0.00^{cd}	7.41	>10	–	1.00 ± 0.02^{b}	<0.41
	LA + CS 1:1	0.34 ± 0.09^{bc}	1.07	0.30 ± 0.02^{b}	6.77	>10	–	1.28 ± 0.01^{c}	<0.39
	LA + CS 1:3	0.26 ± 0.00^{ab}	0.70	0.38 ± 0.01^{d}	6.69	>10	–	2.40 ± 0.01^{e}	<0.58
	CS	0.84 ± 0.01^{d}	–	0.07 ± 0.01^{a}	–	>10	–	>10	–
Decoctions	LA	0.93 ± 0.02^{d}	–	0.09 ± 0.01^{a}	–	7.05 ± 0.02^{c}	–	3.56 ± 0.10^{g}	–
	LA + CS 3:1	0.47 ± 0.07^{c}	0.88	0.31 ± 0.00^{bc}	6.97	>10	–	1.82 ± 0.02^{d}	0.73
	LA + CS 1:1	0.37 ± 0.00^{bc}	0.56	0.32 ± 0.02^{bc}	9.55	>10	–	2.23 ± 0.01^{e}	0.70
	LA + CS 1:3	0.33 ± 0.01^{bc}	0.82	0.33 ± 0.01^{bcd}	7.13	>10	–	3.15 ± 0.04^{f}	0.81
	CS	0.76 ± 0.03^{d}	–	0.09 ± 0.01^{a}	–	>10	–	9.19 ± 0.09^{h}	–
Catechin*		0.07 ± 0.00^{a}		0.62 ± 0.00^{e}		–		–	
BHT*		–		–		1.25 ± 0.08^{a}		0.09 ± 0.00^{a}	

* : Positive control. SE: Synergistic effect (SE < 1: synergistic; SE > 1: antagonistic). 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 test ($p < 0.05$).

of free radicals and inhibition of the free radical chain reactions, thus reducing the generation of lipid peroxidation products (Reed, 2011). Increased oxidative stress and lipid peroxidation are also implicated in the pathogenesis of T2DM, contributing to the initiation and progression of diabetes complications (Kumawat et al., 2013). In this sense, the use of antioxidant beverages, as for example infusions and decoctions of LA and CS mixtures, holds the potential to prevent oxidative stress disorders, including neurogedeneration and diabetes complications (Uttara et al., 2009).

4. Conclusions

In this study, we report for the first time the in vitro antioxidant, neuroprotective, antidiabetic and phenolic composition of herbal mixtures containing infusions and decoctions of flowers of sea lavender (*L. algarvense*, LA) and green tea (*C. sinensis*, CS). Infusions and decoctions of both species had high antioxidant activity, but when applied in combination, the $OH^{\bullet}$ radical-scavenging and anti-lipid peroxidation activities were synergistically improved. Regarding the anti-cholinesterase activity, LA had a stronger inhibition capacity than CS, but their herbal mixtures showed a synergistic effect. CS had high amounts of epicatechin and caffeic acid that can be responsible for its highest α-glucosidase inhibition, however its combination with LA induced an antagonistic effect. In addition, a previous study has shown low toxicity of both species individually (Rodrigues et al., 2016), so their mixtures are not expected to be toxic. Altogether, the data obtained in this work indicate that infusions and decoctions of LA and CS mixtures can be further explored as potential innovative functional beverages able to prevent oxidative stress and lipid oxidation related diseases, and to reduce the progression of neurodegenerative diseases and diabetic complications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2017.12.003.

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Conflict of interest

The authors declare that they have no conflict of interest.

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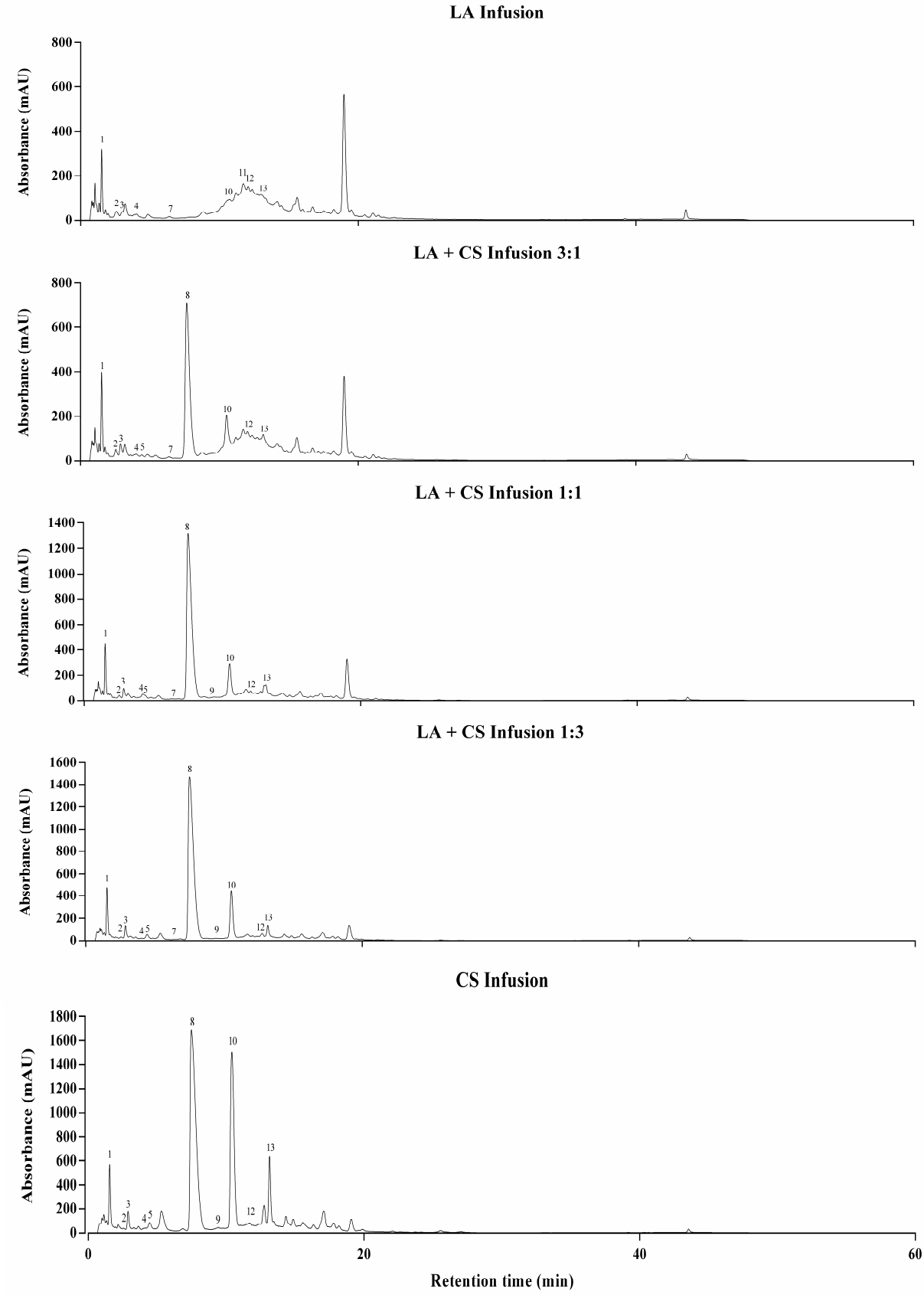
SUPPLEMENTARY MATERIAL



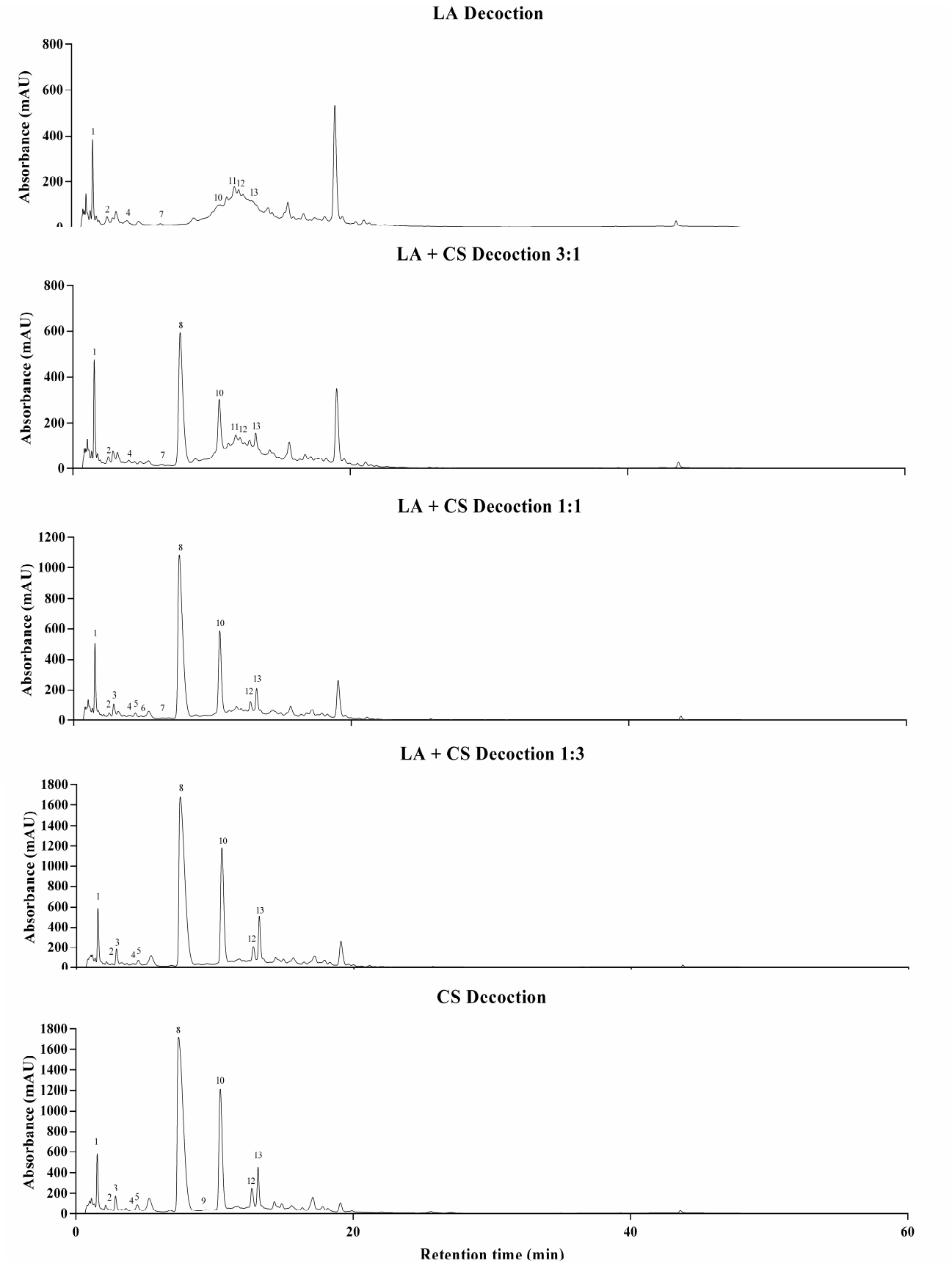


Fig. S1. HPLC–DAD analysis (280 nm) of phenolic compounds in infusions and decoctions of sea lavender (*L. algarvense,* LA), green tea (*C. sinensis*, CS), and their mixtures. Peak numbers refer to the compounds listed in Table 2 of the main text.

Growth performance, *in vitro* antioxidant properties and chemical composition of the halophyte *Limonium algarvense* Erben are strongly influenced by the irrigation salinity

Maria João Rodrigues[1], Ivo Monteiro[2], Viana Castañeda-Loaiza[1], Chloé Placines[1], Maria Conceição Oliveira[3], Catarina Reis[4], Ana D. Caperta[5], Florbela Soares[2], Pedro Pousão-Ferreira[2], Catarina Pereira[1] and Luísa Custódio[1]

[1]Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal

[2]IPMA, Aquaculture Research Station, Olhão, Portugal

[3]Centro de Química Estrutural, Complexo Interdisciplinar, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

[4]Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

[5]Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa, Tapada da Ajuda, Lisboa, Portugal

Abstract

Limonium algarvense Erben (sea lavender) is a halophyte species with potential to provide natural ingredients with *in vitro* antioxidant, anti-inflammatory, neuroprotective and antidiabetic properties. This work reports for the first time the 1) cultivation of sea lavender in greenhouse conditions under irrigation with freshwater (aprox. 0 mM NaCl) and saline aquaculture wastewater (300 and 600 mM NaCl), and 2) the influence of the irrigation salinity on the plant performance (*e.g.* growth, number of produced leaves and flowers), *in vitro* antioxidant properties [radical scavenging activity (DPPH and ABTS), ferric reducing antioxidant power (FRAP), metal chelating properties on copper (CCA) and iron (ICA)], toxicity (*in vitro* on three mammalian cell lines) and chemical composition (determined by LC-ESI-HRMS/MS). The freshwater-irrigated plants had better growth performance than those irrigated with saltwater. Extracts from wild plants, had the highest antioxidant activity, but those from cultivated ones kept high *in vitro* antioxidant properties and interesting chemical profile. The flowers' extracts of plants irrigated with 300 mM of NaCl had the highest antioxidant activities against DPPH, whereas those from freshwater-irrigated plants were more active on ABTS, CCA and FRAP. Most of the extracts showed nil toxicity. The flowers' extracts displayed the highest diversity of compounds, mainly quercetin, apigenin, luteolin, naringenin and their glycoside derivatives. Moreover, their abundance varied with the irrigation salinity. Our results indicate that sea lavender plants can be successfully cultivated in greenhouse conditions and irrigated with freshwater and with irrigation salinity up to 300 mM NaCl; the irrigation salinity influences plant performance, chemical composition and *in vitro* antioxidants properties of produced sea lavender plants and that cultivated plants retain relevant *in vitro* antioxidant properties and chemical components.

Keywords: aquaculture wastewater, dietary supplements, halophyte cultivation, phenolic compounds; sea lavender

4.1. Introduction

Plants are used as a source of health improvement commodities since ancient times, generally in the form of herbal infusions, juices, elixirs, and extracts (Miroddi et al., 2013). Nowadays, botanical nutraceuticals (*e.g*. raspberry ketones, green tea supplements, echinacea, *Garcinia cambogia, Ginkgo biloba*) are also used with the same purposes: to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, and support the structure or function of the body (Nicoletti, 2012; Nasri et al., 2014). These products are sold in different forms, like fresh or dried products, liquid or solid extracts, tablets, capsules, powders, or tea bags (Grand View Research, 2017). Products containing natural ingredients, such as nutraceuticals, have generally easier access to consumers, lower prices and are more effective when compared to prescription drugs, which combined with the growing consumer awareness of the importance of a healthier lifestyle increased the popularity of these products and the demand for natural-based formulations (Nasri et al., 2014; Grand View Research, 2017). This boosted the need to identify and develop innovative and bioactive nutraceutical ingredients, sustaining the projections for the global nutraceuticals market of 578 billion dollars by 2025 (Grand View Research, 2017).

Halophytes are salt-tolerant plants able to grow and prosper under several abiotic stressors, such as high salinity, high UV radiation and drought (Koyro and Huchzermeyer, 2004; Flowers and Colmer, 2008). This is possible due to different physiological and biochemical adaptations, including the production of potent antioxidant molecules, as for example phenolic acids and flavonoids (Ksouri et al., 2012). Besides their vital role in plant protection against oxidative stress, these molecules display important health improving properties (*e.g.* antioxidant, and anti-inflammatory), and are therefore of high interest for different commercial areas (*e.g.* food, pharmaceutical and cosmetics; Flowers et al., 2010; Ksouri et al., 2012; Panche et al., 2016). Halophytes are therefore considered an important pool of natural bioactive ingredients with high added value for several applications, namely as nutraceuticals and dietary supplements (Ksouri et al., 2012). Some species are already commercially exploited for different purposes, as for example *Hippophae rhamnoides* L. (sea buckthorn) as a source of food supplement and cosmetic ingredients (Biotona, 2019; Pipingrock, 2019), *Chenopodium quinoa* Willd. (quinoa) and *Salicornia* spp. (sea asparagus) as food (QuinoaPortuguesa, 2019; Riafresh, 2019) and *Chrithmum maritimum* L. (sea fennel), as a source of cosmetic ingredient (Phytomer, 2019; Seppic, 2019).

The commercial exploitation of a plant must rely on its sustainable cultivation. Halophytes can grow in saline conditions where conventional crops (glycophytes) cannot, such as in integrated multi-trophic aquaculture (IMTA) systems where saline aquaculture effluents are used as irrigation and fertilizers for plant production (Ventura et al., 2015; Waller et al., 2015; Custódio et al., 2017). IMTAs are recommended to accomplish environmental sustainability by biomitigation of aquaculture wastes, while allowing for potential additional incomes by adding crops for commercial purposes, either as food or as sources of bioactive ingredients (Troell et al., 2009). In Europe, the cultivation of some halophytes in IMTA systems was already addressed, including *Aster tripolium* L. (sea aster) and different *Salicornia* species (sea asparagus) (Buhmann et al., 2015; Waller et al., 2015; Custódio et al., 2017).

In our ongoing studies for the commercial valorisation of southwest Portugal selected halophytes, we have identified the endemic species *L. algarvense* Erben (sea lavender) as a potential source of natural ingredients with *in vitro* antioxidant, anti-inflammatory, neuroprotective and antidiabetic properties (Rodrigues et al., 2015, 2016, 2019a). If commercial exploitation of this species will be attempted, its sustainable cultivation and biomass production with the desired properties should be ensured. Therefore, this work had three main goals: 1) Determine if sea lavender plants can be successfully cultivated in greenhouse conditions; 2) Determine the influence of irrigation salinity on plant performance, chemical composition and *in vitro* antioxidant properties of produced sea lavender plants, and 3) Evaluate if cultivated plants retain the *in vitro* antioxidant properties and chemical components of wild plants. For that purpose, sea lavender seeds were collected from the wild and germinated for three weeks, under freshwater irrigation. Obtained plants were then cultivated in greenhouse conditions and irrigated with freshwater (approximately 0 mM NaCl) and aquaculture wastewater in two different dilutions: whole water (600 mM NaCl) and 1:1 dilution (300 mM NaCl). Produced plants were divided in leaves, peduncles and flowers which were used to prepare ethanol extracts by ultrasound-assisted extraction. Extracts were evaluated for *in vitro* antioxidant (radical scavenging and metal chelating) and toxicological properties followed by a chemical characterization of the extracts by liquid chromatography (LC) tandem high-resolution mass spectrometry (HRMS/MS) analysis. Results were compared with those obtained with biomass from sea lavender collected from the wild.

4.2. Materials and methods

4.2.1. Chemicals

Folin-Ciocalteau (F-C) phenol reagent and all solvents used for chemical analysis were bought from Merck (Germany), while Sigma-Aldrich (Germany) provided the 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and butylated hydroxytoluene (BHT). Further chemicals and solvents were supplied by VWR International (Belgium).

4.2.2. Plant material

Sea lavender wild plants were collected in the South of Portugal (Ria de Alvor) in June of 2018 (coordinates: 37°07'34.8"N 8°35'54.9"W). The taxonomical classification was performed by the botanist Dr Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) and a voucher specimen is kept in the herbarium of the XtremeBio laboratory (voucher code XBH1.2). The plants were separated into flowers, peduncles and leaves, dried for 3 days at 40°C, powdered and stored at -20°C until needed. Seeds from sea lavender were collected in the same location in July of 2017 (Ria de Alvor; coordinates: 37°07'34.8"N 8°35'54.9"W).

4.2.3. Greenhouse cultivation

4.2.3.1. Germination

Germination was made in polystyrene plant trays (1 seed per each 3 x 3 cm alveoli, 54 seeds in total), in a 3:1 mixture of peat and perlite (v/v). Seeds were moistened every two days with freshwater (approximately 0 mM NaCl), and germination percentage was recorded weekly, for 3 weeks. Germination was carried out in plastic-greenhouse conditions with a relative humidity of 20–80.2% (min/max) and average temperatures of 7–33.5ºC (min/max.).

4.2.3.2. Plant production

Eight weeks after seeding, plantlets were transplanted to 1 L pots (15 per treatment), containing the same substrate mixture used for germination (3:1 mixture of peat and perlite, v/v), and were irrigated with freshwater during an adaptation period of 4 weeks. Then, plants

were irrigated with progressively increasing concentrations of sterilized saline aquaculture wastewater [from an outdoor tank producing *Sparus aurata* L. (sea bream) and *Dicentrarchus labrax* L. (sea bass)], starting from approximately 50 mM with an increase of 50 mM every two days up to the final concentration. Plants were watered every two days with 100 mL of the irrigation solutions with different NaCl levels, namely approximately 0 (freshwater), 600 mM NaCl (whole water) and 300 mM NaCl (1:1 dilution with freshwater), in each alveolus. The main nutritional components of the used wastewater are summarized in Table S1 of supplementary material. Once a week the freshwater irrigation solution was supplemented with liquid fertilizer (NPK 7-5-6). The photoperiod varied among 13/11 and 14/10 h (day/night) for 1–7 and 8–14 weeks, respectively. The greenhouse temperature and relative humidity conditions, during the 14 weeks of the treatments, are presented in Fig. S1 of supplementary material.

4.2.3.3. Evaluation of growing parameters, fresh (FW) and dry (DW) weights and moisture

After 14 weeks of saline irrigation, the number and height of floral stems were measured, and the leaf number was determined. The plants were then collected and separated into flowers, peduncles and leaves. The leaf surface area was determined (3 leaves per treatment), together with the FW and DW of aerial parts. Moisture was calculated as the difference between FW and DW. Plant survival was also determined. Samples from identical conditions were pooled in a single sample, freeze-dried, powdered and stored at -20°C.

4.2.4. Metabolomics and *in vitro* antioxidant properties

4.2.4.1. Preparation of the extracts

Dried biomass (cultivated and from the wild) was extracted with ethanol by an ultrasound-assisted extraction procedure (1:40, w/v) for 30 min. The extracts were filtered (Whatman nº 4), evaporated under reduced pressure and temperature in a rotary evaporator, weighted, dissolved at the concentration of 10 mg/mL in ethanol, and stored at -20°C.

4.2.4.2. Chemical profile of the extracts by liquid chromatography-tandem high-resolution mass spectrometry (LC-HRMS/MS) analysis

The sea lavender extracts were analysed using an LC-MS with a ProStar 410 autosampler, two 212-LC chromatography pumps, a ProStar 335 diode array detector and a 500-MS ion trap mass spectrometer with electrospray ionisation (ESI) ion source (Varian, Palo Alto, CA, USA). Varian MS Control 6.9 software was used for data acquisition and processing. The separation of the phenolic compounds was performed using a Polaris column (Varian) RP18-A (150 mm × 2 mm I.D., 5 μm particle size, with controlled temperature (35 °C), and the injection of the extract was carried out using a Rheodyne injector with a 20 μL loop. The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The flow rate was 0.25 mL/min, and the gradient as follows: 0–2 minutes A:B (93:7) isocratic, 8 min A:B (75:25) isocratic, 35 min A:B (20:80) isocratic, 40 min A:B (0:100) isocratic, 45 min A:B (93:7) linear. The flow rate was 1 mL/min, and the LC eluent was post-column split in a ratio of 3:1 before being introduced into the mass spectrometer. The mass spectra were acquired in the ESI negative and positive ion modes, in the range from 100 to 1700 u; the optimized parameters were as follows: ion spray voltage, ± 4.9 kV; capillary voltage, 20 and − 60 V; RF loading, 90%. Nitrogen was used as a nebulising and drying gas, at a pressure of 35 and 10 psi, respectively; drying gas temperature, 350 °C. The multistage MS (MS^n) spectra were obtained with an isolation window of 2.0 Da, excitation energy values of 1.0 and 2.5 V and an excitation time of 10 ms. Identification of individual phenolics was performed by their retention times, and both spectroscopic and spectrometric data. The UV spectra for the compounds were compared with the available library of previously recorded UV spectra for a wide range of compounds.

4.2.4.3. Radical scavenging activity (RSA) on $DPPH^{\bullet}$ and $ABTS^{+\bullet}$

Samples were tested for RSA against the DPPH and ABTS radicals at concentrations ranging from 10 to 1000 μg/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 a percentage of inhibition, relative to a control containing ethanol in place of the sample, and as half maximal inhibitory concentration (IC_{50} values, μg/mL), when possible.

4.2.4.4. Ferric reducing antioxidant power (FRAP)

The ability of the extracts to reduce Fe^{3+} 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 IC_{50} values (µg/mL), when possible.

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

ICA and CCA were tested on samples at different concentrations (10 – 1000 µg/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 a percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as IC_{50} values (µg/mL), whenever possible.

4.2.5. Cell culture and cytotoxicity of the extracts

The murine RAW 264.7 macrophages, the human embryonic kidney (HEK) 293, and the human hepatocellular carcinoma HepG2 cell lines were respectively provided by the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal), the Functional Biochemistry and Proteomics, and the Marine Molecular Bioengineering groups (Centre of Marine Sciences, Portugal).

The RAW 264.7 cells were maintained in RPMI 1640 culture media, while HEK 293 and HepG2 cell lines were cultured in DMEM media, both supplemented with 10% heat-inactivated FBS, 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL) / streptomycin (50 µg/mL). All cell lines were kept at 37ºC in moistened atmosphere with 5% CO_2. Exponentially growing cells were plated in 96-well tissue plates at a density of 1 x 10^4 cells/well (RAW 264.7) and 5 x 10^3 cells/well (HEK 293 and HepG2), followed by 24 h incubation. The extracts were then applied at the concentration of 100 µg/mL for 72 h. Control cells were treated with DMSO at the highest concentration used in test wells (0.2%), and cell viability was determined by the MTT colorimetric assay (Biotek Synergy 4), as described previously (Rodrigues et al., 2014). Results were expressed in terms of cellular viability (%).

4.2.6. Statistical analysis

Results were expressed as mean ± 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 the Tukey HSD test ($P < 0.05$). All statistical analyses were performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation). The IC_{50} values were calculated by the sigmoidal fitting of data using the GraphPad Prism v. 5.0 program.

4.3. Results and discussion

4.3.1. Germination and plant growth performance

Despite the high commercial potential of several halophytes and the need for economically, socially and environmentally viable production systems, research regarding the cultivation of selected species is still limited when compared to glycophytes (Ventura et al., 2015). Moreover, it is known that halophyte plant growth and biochemical profile of obtained biomass can be influenced by agronomic conditions, including the salinity irrigation, and can be optimized to produce biomass with desired functional properties (Boestfleisch et al., 2014). Thus, this work attempted the greenhouse production of *L. algarvense* and evaluated the influence of saline irrigation on the growth performance, *in vitro* antioxidant and chemical properties of the produced plants.

Halophyte seeds' germination is affected by, for example, salinity and temperature (Khan and Gul, 2006), and for several *Limonium* species, such as *L. cossonianum* Kuntze, *L. tabernense* Erben and *L. supinum* (Girard) Pignatti, highest germinations rates are usually obtained with freshwater treatments (Giménez et al., 2013; Delgado Fernández et al., 2016; Melendo and Giménez, 2019). Therefore, in this work, sea lavender seeds were germinated using freshwater irrigation only. The first seeds germinated after 3 days, and at the end of the 1st week we observed a germination percentage of 7.4%, which increased to 51.8 and 81.5% in 2nd and 3rd weeks, respectively (Fig. 4.1). In a previous work, the germination rate (84%) of *L. tabernense* treated with freshwater and temperatures above 30ºC (Delgado Fernández et al., 2016), was close to that obtained by *L. algarvense.* Also, *L. supinum* seeds also had high germination rates (98%) when treated with freshwater in all temperature conditions (20/10 – 35/25ºC) (Melendo and Giménez, 2019). Likewise, *L. cossonianum* exhibited 90% of germination rate with freshwater soaking (Giménez et al., 2013).

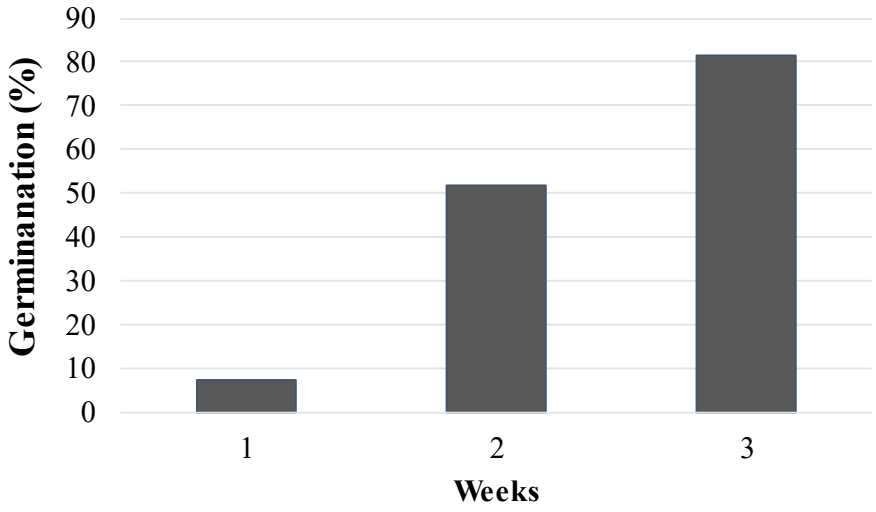


Fig. 4.1. Percentage of germination for 3 weeks.

After transplantation and acclimatization, plants were submitted for 14 weeks to different irrigation treatments, including approximately 0 (freshwater), 300 and 600 mM NaCl of saline aquaculture wastewater. Afterwards, the above-ground plant organs were harvested and divided into flowers, peduncles and leaves. All plants from all the irrigation conditions survived until the end of the cultivation period. However, plants irrigated with 600 mM NaCl were not able to produce flower stems and flowers. The moisture and dry matter contents are depicted in Fig. 4.2. Leaves from freshwater-irrigated plants had the highest moisture content (79.8%), amongst all treatments. Additionally, leaves' moisture level decreased with increasing salinity, whereas no significant variation was observed in flowers and peduncles (60.5 – 55.5% and 60.4 – 54.1%, respectively; $P < 0.05$). Figure 4.3 shows the growth performance parameters of sea lavender for each irrigation salinity treatment. The highest number (2.3) and height (36.4 cm) of the floral stems was obtained in freshwater-irrigated plants, which significantly decreased when plants were irrigated with saline aquaculture wastewater at 300 mM ($P < 0.05$). The same tendency was found on the leaves, concerning its number and surface area (54.7 and 11.6 cm^2, respectively; Fig. 4.3).

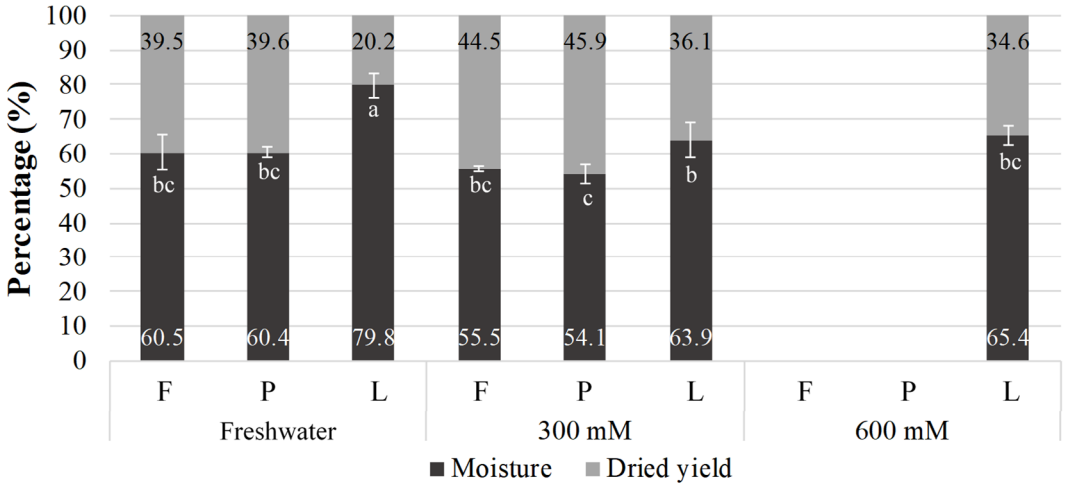


Fig. 4.2. Moisture and dried matter contents (%) of flowers (F), peduncles (P) and leaves (L) of sea lavender plants irrigated with freshwater and aquaculture wastewater at 300 and 600 mM NaCl concentrations. Columns labelled with different letters are significantly different at $P < 0.05$ (Tukey HSD test).

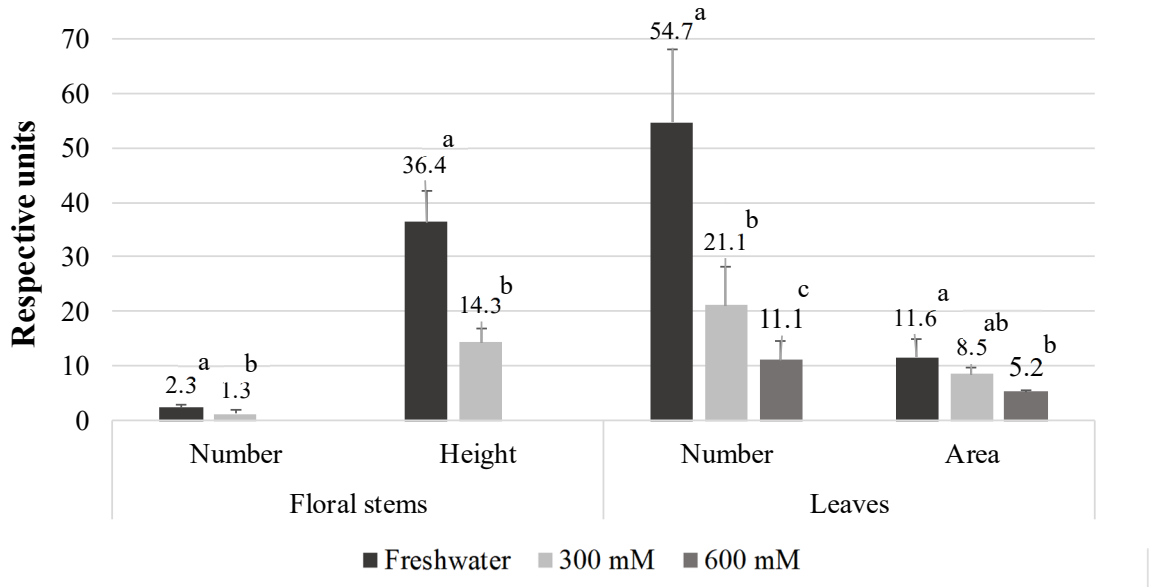


Fig. 4.3. Number and height (cm) of floral stems, and number and area (cm^2) of leaves of sea lavender plants irrigated with freshwater and aquaculture wastewater at 300 and 600 mM NaCl concentrations. For each group, columns labelled with different letters are significantly different at $P < 0.05$ (Tukey HSD test).

Although it is theoretically assumed that halophytes grow better under saline conditions (Panta et al., 2014), there are several reports of different species exhibiting better growth performance under non-saline conditions, similar to our results. For example, this effect was observed on cultivated *Inula crithmoides* L., *Plantago crassifolia* Forssk. and *Medicago marina* L. (Grigore et al., 2012), as well as with *Cakile maritima* Scop. (Ksouri et al., 2007) and *Polygonum maritimum* L. (Rodrigues et al., 2019b). These observations may be related to other restrictive aspects besides salinity, as for example, accessibility to light, nutrients, and water, which can influence the interspecific competition (Grigore et al., 2012). Thus, to avoid competition with glycophytes in non-saline habitats, halophytes preferentially colonize saline environments, where they have a competitive advantage due to their salt tolerance (Grigore et al., 2012). In turn, salinity compromises some plant functions, leading to hydric stress, reduced plant biomass, impaired photosynthesis, leaf damage, and nutrients deprivation (Koyro et al., 2008). These problems, coupled with high toxicity of sodium and chloride ions, may explain the decreased plant growth and consequent reduced leaves number and surface area observed with sea lavenders irrigated with saline water (Ali et al., 2004). The same effects were previously reported with other halophytes, namely *Atriplex hortensis* L. and *C. maritima* (Ksouri et al., 2007; Kachout et al., 2009). Similar to our results, salinity also reduced the floral stems and flowers number of C. *maritimum* (Ventura et al., 2014). However, flowering was stimulated in saline conditions on the halophytes *P. crassifolia* and *Suaeda salsa* (Grigore et al., 2012; Guo et al., 2018),

Regardless the reduced growth and flowering, sea lavender plants irrigated at 300 mM of NaCl were able to complete their life cycle (produce flowers and seeds), which indicates that this species can be cultivated under saline irrigation up to that NaCl level. Additional work is however needed to optimize the cultivation conditions to increase productivity, for example, by optimizing the substrate and nutritional supplementation of the irrigation water (Buhmann et al., 2015).

4.3.2. Chemical composition

The metabolic profile of the sea lavender ethanol extracts was established by LC-ESI-HRMS/MS, and the list of proposed compounds is presented in Table 4.1. A total of 52 compounds, mainly flavonoids and its glucoside derivatives, were tentatively identified in the flowers, peduncles and leaves, but some were only detected in one specific plant organ, specific irrigation salinity, in wild or cultivated plants.

The flowers had the highest number of compounds (twenty-one) only detected in this organ, which included digalloyl-hexoside (**3**), hex-3-en-1-olxylopyranosyl -(1-6)-glucopyranoside (**7**), epigallocatechin gallate (**9**), licoagroside B (**10**), isorhamnetin-3-O-rutinoside (**17**), methyl licoagroside B (**19**), quercetin-tetramethyl ether-dihydroxyethyl-fructopyranose (**20**), quercetin-O-hexoside (**22**), 2'-C-methyl myricetin-3-rhamnoside-5''-galloyl (**23**), apigenin-7-O-glucoside (**29**), apigenin-7-O-glucuronide (**31**), luteolin-O-glucoside (**33**), luteolin-O-rhamnoside (**35**), apigenin derivative (**37**), 4'-methyl eriodictyol-galloyl-rhamnose (**38**), eriodictyol (**39**), naringenin derivative (**42**), luteolin (**44**), dihydrokaempferol (**45**), apigenin (**49**) and naringenin (**50**). Only seven compounds were only present in the leaves, namely glucosyringic acid (**4**), eriodyctiol-O-glucoside (**11**), myricitin-3-O-rutinoside (**12**), rutin (**16**), myricetin-O-acetyl-hexose (**25**), quercetin-hexoside derivative (**34**), myricetin-galloyl-acetyl deoxyhexose (**40**). Five molecules were only present in the peduncles, namely galloyl glucose derivative (**5**), sinapyl alcohol sulfate (**6**), galloylhexoside derivative (**14**), tryptophan (**28**) and feruloyltyramine (**43**).

Phenolic compounds are implicated in several plant-environment interactions (*e.g.* against herbivory, UV-radiation, pollination) and their presence on the different plant organs varies according to their biochemical/physiological functions (Pagare et al., 2015). For example, the flowers are usually richer in flavonoids, which contribute to pigmentation that is implicated on the pollination process (Pichersky and Gang 2000; Atmani et al. 2009; Iwashina, 2015). The prevalence of flavonoids in the sea lavender flowers is most likely related to these functions.

Table 4.1. LC-ESI-HRMS/MS tentative identification of metabolites present in ethanol extracts of sea lavender organs (flowers, peduncles and leaves) irrigated with freshwater, two dilutions of aquaculture wastewater (300 and 600 mM), in comparison to wild plants (WT). For distinguishing amongst low, medium or high abundance the symbols +, ++ and +++ were used, respectively.

ID	Rt	Proposed structure	$[M-H]^-$	MS^2 (m/z)	Proposed compound	Flowers			Peduncles			Leaves			
						Fresh water	300 mM	WT	Fresh water	300 mM	WT	Fresh water	300 mM	600 mM	WT
1	3.20	C12H22O11	341.1094	179, 119	Sucrose or isomeric structures	+	+	+	+	+	+	+++	++	+	+++
2	3.50	C13H16O10	331.0679	169, 125	Galloyl-hexoside	+	+	+	+	+++		+	+	+	+
3	5.70	C20H20O14	483.07938	331	Digalloyl-hexoside	+	+	+++							
4	5.70	C15H20O10	359.0982	179, 153	Glucosyringic acid							+++	+	+++	
5	6.9	C20H22O12	453.104	313, 169	Galloyl glucose derivative				+++						
6	7.4	C11H14O4SO3	289.0386	209, 149	Sinapyl alcohol sulfate				+++	+					
7	7.60	C17H30O10	393.1778	205, 179	Hex-3-en-1-olxylopyranosyl -(1-6)-glucopyranoside	+	+++								
8	7.80	C28H24O17	631.0944	479, 316	Myricetin-3-*O*-(2"-*O*-galloyl) glucoside		+	+	+	+	++	+	++	++	+++
9	7.90	C22H18O11	457.0779	305	Epigallocatechin gallate	+++	+	++							
10	8.00	C18H24O12	431.1192	285, 225	Licoagroside B	+++	+++	+							
11	8.00	C21H22O11	449.1095	287, 269, 259	Eriodyctiol-*O*-glucoside							+			+++
12	8.00	C27H30O17	625.1415	316	Myricitin-3-*O*-rutinoside							++	+++	++	+

13	8.20	C21H20O13	479.0836	316, 217, 259	Myricitin-3-*O*-glucoside	+	+	+	+	+	+++	+	+	+	+
14	8.50	C22H22O12	477.1046	433, 313, 169	Galloylhexoside derivative				+++						
15	8.60	C28H24O16	615.0985	463, 301, 300	Quercetin-O-(galloy)-glucoside					+	+++				+++
16	8.60	C27H30O16	609.146	463, 301, 300	Rutin										+++
17	8.70	C28H32O16	623.1618	314, 299	Isorhamnetin-3-O-rutinoside	+++	+++	+							
18	8.70	C20H10O12	449.0729	316	Myricetin-3-*O*-pentoside						+	+++	++	++	+
19	8.80	C19H26O12	445.1352	285, 225	Methyl licoagroside B	+++	+++	+							
20	8.8	C27H34O15	597.1825	459, 417, 387, 357	Quercetin-tetramethyl ether-dihydroxyethyl-fructopyranose	+++		+							
21	8.90	C21H20O12	463.0889	316, 217	Myricitin-3-*O*-rhamnose	+	+	++	+	+	++	++	++	++	+++
22	9.30	C21H20O12	463.0876	301	Quercetin-*O*-hexoside	+	++	+++							
23	9.30	C29H26O16	629.1152	479, 316	2'-C-methyl myricetin-3-rhamnoside-5"-galloyl	+++	+								
24	9.40	C28H24O15	599.1045	447, 316	Myricetin -ethyl acetoacetate-galloyl -			+			+++				
25	9.40	C23H22O14	521.0939	316, 217	Myricetin-O-acetyl-hexose							+++	+	+++	
26	9.50	C9H10O5	197.044	124	Syringic acid			+++	+						
27	9.80	C21H20O11	447.0935	301, 300, 271, 255	Quercetin-3-*O*-rhamnoside	+++	+++	++	+	+		++	+	++	++

28	9.8	C13H14N2O3	245.0932	203, 142	Tryptophan				+++	+					
29	9.90	C21O20O10	431.0983	269, 268	Apigenin-7-O-glucoside	+	+++	+++							
30	9.90	C23H22O13	505.0995	316, 217	Myricetin-*O*-acetyl-deoxyhexose		+					+++	+++	++	+
31	10.10	C21H18O11	445.0778	269	Apigenin-7-*O*-glucuronide	++	+++	+							
32	10.2	C28H24O16	615.0998	317	Myricetin-*O*-(galloyl)-deoxyhexose			+	+	+	++	+++	+++	++	+
33	10.3	C21H20O11	447.0935	285, 284, 255	Luteolin-*O*-glucoside	+	+++	+							
34	10.30		583.11104	463, 300	Quercetin-hexoside derivative										+++
35	10.5	C21H20O10	431.0977	285, 284, 255	Luteolin-O-rhamnoside	++	+	+++							
36	10.70	C15H10O8	317.0304	217, 179, 151	Myricetin			++	+		+++				
37	11.00	C29H26O14	597.1247	269	Apigenin derivative	+	+++								
38	11.00	C28H24O15	599.1051	447, 301, 269	4'-methyl eriodictyol-galloyl-rhamnose			+++							
39	11.20	C15H12O6	287.0555	125	Eriodictyol	+++	+								
40	11.40	C20H26O17	657.1102		Myricetin-galloyl-acetyl deoxyhexose							+++	+	+	
41	11.50	C20H22O9S	437.0907	357, 342, 151	Pinoresinol sulphate	++	++	+	++	+	+	+	++	+	+++
42	11.50	C29H28O14	599.1404	453, 271	Naringenin derivative	+++	+++	+							
43	11.7	C18H19NO4	312.1247		Feruloyltyramine				+++						

44	12.20	C15H20O6	285.0404	199, 175, 151, 133	Luteolin	+	+++	+++								
45	12.30	C15H12O6	287.0563	199, 151, 135	Dihydrokaempferol	+++	+++	+								
46	12.3	C15H10O7	301.0356	151	Quercetin											+++
47	12.30		547.1472	300	Quercetin derivative											+++
48	13.30	C18H22O5	327.2178		Trihydroxy-10,15-octadecadienoic acid	+++	+++	+	+	+	+	+	+	+	+	
49	13.50	C15H10O5	269.0458	227, 151, 117	Apigenin	+	+++	+								
50	13.70	C15H12O5	271.0612	187, 151, 119	Naringenin	+	+	+++								
51	14.10	C18H34O5	329.2337		Trihydroxy-10-octadecenoic acid	+	++	+	+++	++	++	+	+	+	+	
52	14.3	C13H24O3SO3	307.1223		Oxo-tridecanoic acid sulphate				+++		+					

Amongst all the compounds, a few were only detected in the wild plants' extracts, mainly quercetin (**46**) and several of its derivatives (**16**, **34**, **47**). A higher number of compounds was identified only in the cultivated plants, mostly the flavonoids eriodictyol (**39**) and some flavonoid glycosides (**23**, **25**, **37**). Besides, galloyl glucose (**5**) and galloyl hexoside (**14**) derivatives were only present in flowers of freshwater-irrigated plants. Moreover, the relative abundance of several compounds fluctuated with the irrigation salinity. For instance, in the extracts from leaves, the levels of the molecules **1**, **18**, **30**, **32** and **40** decreased with increasing irrigation salinity, reaching the highest content in plants irrigated with freshwater. Conversely, compound **8** exhibited the opposite trend. Regarding the flowers' samples, more compounds had shown a variation with this parameter, for example, the molecules **7**, **22**, **29**, **31**, **33**, **37**, **44**, **49** and **51** increased, while **9**, **23**, **35** and **39** decreased with the increasing irrigation salinity. In turn, compounds **6**, **28** and **41** were detected in higher abundance in the peduncles from plants irrigated with freshwater. This variation suggests that some compounds may be produced as a part of a salt-stress resistance mechanism. For example, an increase in the concentration of less hydroxylated flavonoids, such as apigenin (**49**) and luteolin (**44**) have been reported to be linked to an enhanced salinity tolerance in some glycophytes, such as endive or rice (El-Shafey and Abdelgawad, 2012; Mekawy et al., 2018). This change for less hydroxylated forms may be associated with an altered activity or/and expression of oxidases and dehydrogenases enzymes (*e.g.,* flavonol synthase and flavonoid 3′-hydroxylase) under stress conditions (Fini et al., 2011; Chapman et al., 2019). For instance, the reduced prevalence of myricetin and its derivatives in the leaves of wild and saline-irrigated plants, coupled with a higher occurrence of more oxidatively stable quercetin and its derivatives found in wild plants, could be related with a more stressful environment, with higher UV-radiation and temperature (in the wild), and increasing salinity of the irrigation solution (in the cultivated plants) (Csepregi and Hideg, 2018).

Overall, just three compounds were previously reported in this species, namely epigallocatechin gallate (**9**), syringic acid (**26**) and apigenin (**49**) in methanol and infusions from flowers of the same species collected from the wild (Rodrigues et al., 2015, 2016, 2019). However, several of the compounds detected in the present work have already been described in other species of the genus *Limonium*. Eriodictyol (**39**) and luteolin (**44**) were previously detected in ethyl acetate extracts from *L. bondueli* aerial organs (Benaissa et al., 2013) and from *L. bicolor* flowers (Chen et al., 2017). The latter species was also described to contain quercetin (**46**), rutin (**16**) and quercetin-3-*O*-rhamnoside (**27**) (Chen et al., 2017). Medini et al. (2017) reported the occurrence of feruloyltyramine (**43**), dihydrokaempferol (**45**) and pinoresinol in *L.*

densiflorum ethanol shoot extract (Medini et al., 2017). In turn, apigenin-7-O-glucoside (**29**), luteolin-O-glucoside (**33**) and naringenin (**50**) were previously detected in ethanol extracts from *L. insigne* inflorescence stems, leaves and roots (Ortuño et al., 2018). Myricetin (**36**) was already reported in ethanol extracts from aerial parts of *L. caspium* (Willd) (Gadetskaya et al., 2015).

4.3.3. Antioxidant properties

Several human health problems, including coronary diseases, cancer, age-related degenerative brain disorders, Type 2 diabetes, chronic inflammation, as well as the normal ageing process, can originate or be exacerbated by oxidative stress states (Liguori et al., 2018), which mean the occurrence of an imbalance amongst cellular antioxidant defence systems and the production of reactive oxygen species (ROS) and free radicals. This situation causes the impairment of vital cellular molecules, such as DNA, proteins and lipids, which leads to the development of the diseases mentioned above (Kohen and Nyska, 2002). In this context, antioxidant ingredients can stabilize or deactivate free radicals avoiding and diminishing cellular injury contributing to health maintenance. In this work, the *in vitro* antioxidant properties of sea lavender ethanol extracts were assessed using five different methods, namely radical scavenging activity of DPPH and ABTS, metal chelation of iron (ICA) and copper (CCA), and ferric reducing antioxidant power (FRAP). Results are presented in Table 4.2.

Generally, the wild plants showed the highest antioxidant activity, and flowers showed the lowest IC_{50} value against the DPPH radical (IC_{50} = 123 µg/mL), whereas the peduncles were the most active on ABTS, and on the CCA and FRAP assays (IC_{50} = 143, 320 and 38 µg/mL, respectively). Amongst the cultivated plants, the flowers irrigated with 300 mM NaCl had the best capacity to scavenge DPPH, (IC_{50} = 276 µg/mL), but the flowers from freshwater-irrigated plants had the uppermost RSA towards ABTS, CCA and FRAP (IC_{50} = 467, 768 and 117 µg/mL, respectively). Concerning the leaf extracts, the FRAP decreased with increasing irrigation salinity, and the best activity was exhibited by the extracts from freshwater-irrigated sea lavender (IC_{50} = 209 µg/mL). Although a different pattern was found towards DPPH, the freshwater-irrigated plants had the lowest AA, presenting the lowest IC_{50} value at 300 mM of NaCl (647 µg/mL), which decreased at 600 mM-irrigated plants.

Table 4.2. *In vitro* antioxidant activities of ethanol extracts of sea lavender organs (flowers, peduncles and leaves) irrigated with freshwater and two dilutions of aquaculture wastewater (300 and 600 mM), and from plants collected from the wild. Results are expressed as IC_{50} values (μg/mL).

Extract	Treatment	Plant organ	DPPH	ABTS	CCA	FRAP
Ethanol	Freshwater	Flowers	414 ± 11^{b}	467 ± 19^{b}	768 ± 32^{d}	117 ± 1^{c}
		Peduncles	639 ± 9^{c}	843 ± 35^{d}	-	273 ± 7^{e}
		Leaves	-	-	-	209 ± 7^{d}
	300 mM	Flowers	276 ± 4^{ab}	657 ± 15^{c}	-	269 ± 9^{e}
		Peduncles	692 ± 11^{c}	898 ± 30^{d}	-	205 ± 5^{d}
		Leaves	647 ± 25^{c}	-	-	325 ± 17^{f}
	600 mM	Leaves	946 ± 13	-	-	376 ± 9^{g}
	Wild	Flowers	123 ± 3^{a}	199 ± 16^{a}	348 ± 6^{b}	81 ± 6^{bc}
		Peduncles	373 ± 6^{b}	143 ± 4^{a}	320 ± 2^{b}	38 ± 3^{a}
		Leaves	149 ± 5^{a}	625 ± 62^{c}	627 ± 7^{c}	74 ± 10^{ab}
Positive control*			111 ± 9^{a}	142 ± 11^{a}	171 ± 9^{a}	-

-: activity lower than 50% at 1 mg/mL. *Positive controls: RSA of DPPH and ABTS (BHT), and CCA (EDTA). Values represent the mean ± 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).

Flavonoids and their derivatives, the most identified compounds in sea lavender extracts, have diverse biological properties (*e.g.* antiallergenic, antiviral, anti-inflammatory), but their most relevant capacity is as antioxidants (Nijveldt et al., 2001, Kumar and Pandey, 2013). Thus, flavonoid-rich ingredients, such as the sea lavender flower extracts, may neutralize free radicals leading to decreased cellular damage, delaying disease development and improving health (Nijveldt et al., 2001). The highest flavonoids diversity in the flowers may be related to the higher antioxidant properties observed in the extract from this organ. Moreover,

the highest activity found in the wild plants suggests that salinity may not be the only stressor that influences the production of antioxidants, such as flavonoids. In fact, the production and accumulation of high levels of antioxidant molecules may result from a combination of several factors, as for example high UV-radiation, temperature variation between day and night and/or herbivory, as well as salinity (Ramakrishna and Ravishankar, 2011; Yang et al., 2018). Moreover, the accumulation of flavonoids may also be influenced by the developmental stage, species, cultivars and post-harvest processes (Cetinkaya et al., 2017).

Furthermore, the different activity patterns obtained in the various methods may be due to the different mechanisms of reaction of the extracts' compounds with the different oxidizing agents, *i.e.*, the same molecule may react in a distinct way with the different reactive species (Dai and Mumper, 2010; Niki and Noguchi, 2000). In addition, since oxidative stress includes a wide range of reactive species, it is also important to use diverse methods to fully evaluate the antioxidant potential of a sample (Badarinath et al., 2010; Niki, 2010).

Previous work on sea lavender plants collected from the wild described the high *in vitro* antioxidant potential of methanol extracts from different plant organs, especially from flowers, with significant RSA towards DPPH and ABTS, and also high copper chelating and ferric reducing properties (Rodrigues et al., 2015). Other work focused on the antioxidant properties of infusions and decoction from flowers and suggested that these may have potential as functional beverages (Rodrigues et al., 2016). In this work the irrigation salinity influenced the antioxidant capacity of obtained extracts, similar to what was already reported for other halophytes. For example, the activity of extracts from *Sesuvium portulacastrum* (L.) L. was dependent on the plant organ, for instance, leaf and root had increased activity with increasing salinity levels, whereas stems displayed a reduced antioxidant activity (Slama et al., 2015). Also, the same species showed an increased activity when irrigated with concentrations up to 400 mM NaCl, followed by a decrease up to 800 mM of NaCl irrigation (Slama et al., 2017). In turn, the antioxidant properties of extracts from six-week-old plants of *Bruguiera cylindrica* (Linnaeus) Blume*, Tripolium pannonicum* L. and *Lepidium latifolium* L. was not influenced by irrigation with saltwater (Boestfleisch et al., 2014). On the contrary, the antioxidant activity of the species *Atriplex halimus* L. was enhanced with increasing salinity (200 – 400 mM NaCl) (Bendaly et al., 2016). In the case of *C. maritima* a different pattern was observed according to the different seeds' origin, for example, plants originated from seeds from one location (Tabarka) showed reduced antioxidant activity with higher NaCl concentrations, whereas those from seeds collected from the other location (Jerba) showed no variation with salinity levels (Ksouri et al., 2007). Another study, comparing the antioxidant properties of *C. maritima* at

two distinct stages (vegetative and flowering), observed a peak in the antioxidant properties on the vegetative period, but during the flowering stage, the activity fluctuated amongst the different soil salinities (Mansour et al., 2018). These facts suggest that the antioxidant properties of produced biomass from halophyte species are influenced by a set of conditions, including not only salinity but also seed origin, stages of growth (vegetative/flowering) and plant organ. A defined pattern cannot be found amongst the reported studies for different halophytes, proposing that these adaptations may be species-specific.

4.3.4. Toxicological evaluation

Natural products are generally acknowledged to be safer than the synthetic ones (Karimi et al., 2015). However, plants can be intrinsically toxic due to their chemical composition, (Nasri and Shirzad, 2013) and therefore, it is of upmost importance to ensure their safety for potential consumers. The toxicity of botanical ingredients is usually determined by their cytotoxic effects on mammalian cell lines as it is suggested to correlate in a positive way to *in vivo* models (Carballo et al. 2002; Parra et al. 2001; Blazka and Hayes, 2001). Following this, the sea lavender ethanol extracts were evaluated for their *in vitro* cytotoxicity on three mammalian cell lines: murine RAW 264.7 macrophages, human embryonic kidney (HEK) 293, and human hepatocellular carcinoma HepG2 cells (Fig. 4). Samples allowing cellular viabilities higher than 80% were considered non-toxic (Rodrigues et al. 2014, 2016).

All the extracts had no toxicity against HEK 293 and HepG2 cell lines, and most of them exhibited cellular viabilities above 100% (Fig. 4.4A e 4.4B). Regarding HEK 293 cells, extracts from flowers of freshwater-irrigated plants had the highest values of 147 and 142% of cell viability, respectively (Fig. 4.4A). Similarly, several samples increased HepG2 cells viability over 150%, such the flowers extract from plants irrigated with 300 mM of NaCl and from those collected from the wild (185 and 176%, respectively; Fig. 4.4B). However, a few extracts reduced the viability of RAW 264.7 macrophages, namely the wild leaves and peduncles (67.4 and 74.3%, respectively), leaves of plants irrigated with 300 mM aquaculture wastewater (75.4%) and peduncles of freshwater-irrigated plants (78.2%). The remaining samples were considered non-toxic towards RAW 264.7 macrophages (Fig. 4.4C).

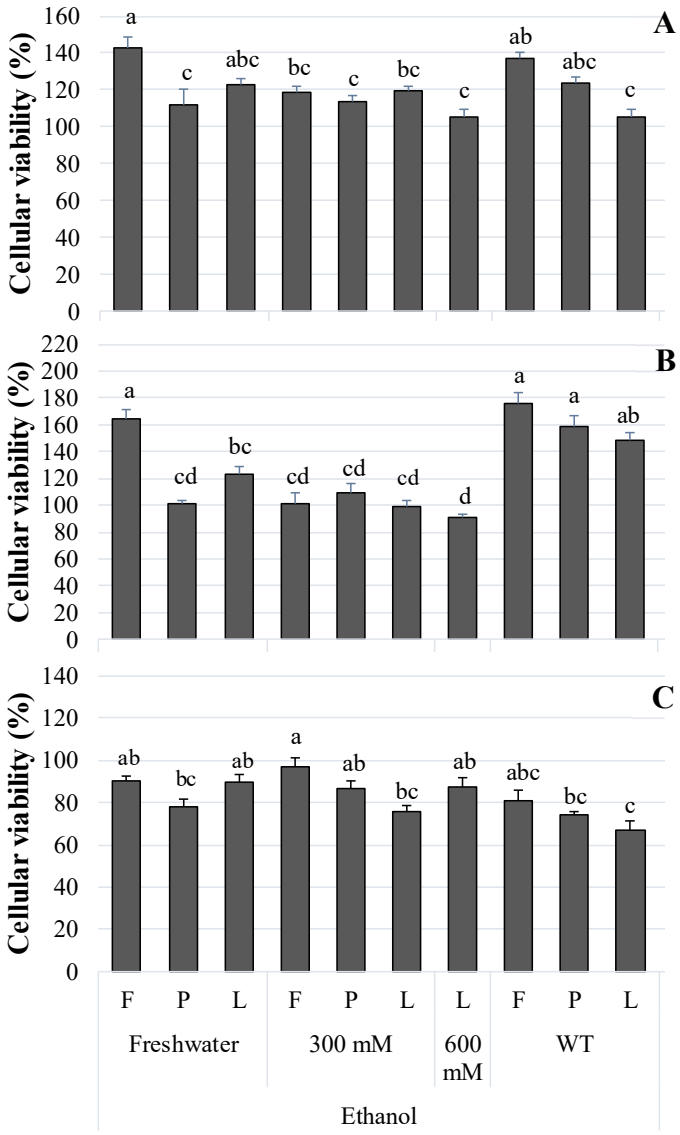


Fig. 4.4. Cytotoxicity of ethanol extracts of sea lavender organs (F – flowers; P – peduncles; and L - leaves) from plants irrigated with freshwater and two concentrations of aquaculture wastewater (300 and 600 mM), and plants collected from the wild (WT) on HEK 293 (**A**), HepG2 (**B**), and RAW 264.7 (**C**) cell lines. Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate (n = 9). Columns marked by different letters are significantly different according to the Tukey HSD test ($P < 0.05$).

The increased cellular viability observed after the application of sea lavender extracts could have two different explanations. First, it is important to keep in mind that the MTT assay is a colorimetric assay that measures the activity of mitochondrial NAD(P)H-dependent cellular oxidoreductase enzyme to reduced MTT (Aslantürk, 2017). Therefore, an increase in cellular viability may be the result of increased enzymatic activity or higher cellular proliferation.

Moreover, and despite being described with anti-tumour effects, flavonoids have shown lower or nil-toxicity to non-tumoral cells (Nijveldt et al., 2001), and in fact several flavonoids, such as apigenin, quercetin, naringenin and rutin (present in sea lavender extracts) have been reported with hepatoprotective effects, *i.e.*, protect against induced hepatotoxicity in HepG2 cells, and also with liver regenerative properties (Tapas et al., 2008; Kumar and Pandey, 2013). This may also explain the increased cellular viability resulting from the application of sea lavender extracts. Besides, the reduced cell viability induced by the wild plants rather than greenhouse produced ones, can be associated with the occurrence of some toxic compounds (*e.g.* alkaloids), which are usually synthesized by wild plants for protection against herbivores (Stamp, 2008).

Infusions and decoctions made from sea lavender flowers were identified previously as a potential antioxidant and anti-inflammatory functional beverages, and the toxicological assessment of these formulations was also reported against mammalian cell lines (Rodrigues et al., 2016). Similar to the present results, all samples were non-toxic at 100 µg/mL, and some increased HepG2 and microglial (N9) cells viability above 100% (Rodrigues et al., 2016). Since the in vitro toxicity against mammalian cell lines is positively correlated with in vivo toxicity on mice (Garle et al., 1994; Di Nunzio et al., 2017), the results of our study indicate that extracts from the cultivated sea lavender may be considered as safe for application as nutraceutical ingredients. Nevertheless, additional experiments are needed to confirm these preliminary tests.

4.4. Conclusions

In this study, we report for the first time the cultivation of sea lavender in greenhouse conditions under irrigation with saline aquaculture wastewater (at two NaCl levels), and the influence of that salinity on plant growth, and on the *in vitro* antioxidant, toxicological and chemical properties of ethanol extracts from aerial organs of produced sea lavenders, compared to those from wild plants. Overall, the obtained results suggest that:

1) Sea lavender plants can be successfully cultivated in greenhouse conditions and irrigated with freshwater and with irrigation salinity up to 300 mM NaCl. The percentage of germination after 3 weeks was 81% and sea lavender plants irrigated with freshwater and at 300 mM NaCl were able to complete their life cycle (produce flowers and seeds).

2) The irrigation salinity influences plant performance, chemical composition and *in vitro* antioxidants properties of produced sea lavender plants. Freshwater irrigated plants exhibited better growth performance, plants irrigated with 300 mM of NaCl were able to

complete the life cycle while those irrigated with 600 mM NaCl were not able to produce flower stems and flowers; the irrigation salinity decreased plant growth, including the number of flowers and leaves. The *in vitro* antioxidant properties and the chemical composition were maintained under saline and non-saline irrigation.

3) Cultivated plants retain the *in vitro* antioxidant properties and chemical components of wild plants. Although extracts from wild plants had generally a higher RSA on the DPPH and ABTS radicals, copper chelating and ferric reducing activities than those from cultivated ones, a significant antioxidant capacity was still observed in extracts from cultivated plants. Flavonoids were the main compounds present in the extracts and their presence varied within the source of biomass (wild/cultivated). However, cultivated plants were still rich in bioactive molecules.

Therefore, sea lavender could be candidate for commercial production in saline conditions, for example in IMTA system using diluted aquaculture wastewater, or in other systems using brackish water for irrigation, to be used as a source of bioactive ingredients.

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SUPPLEMENTARY MATERIAL

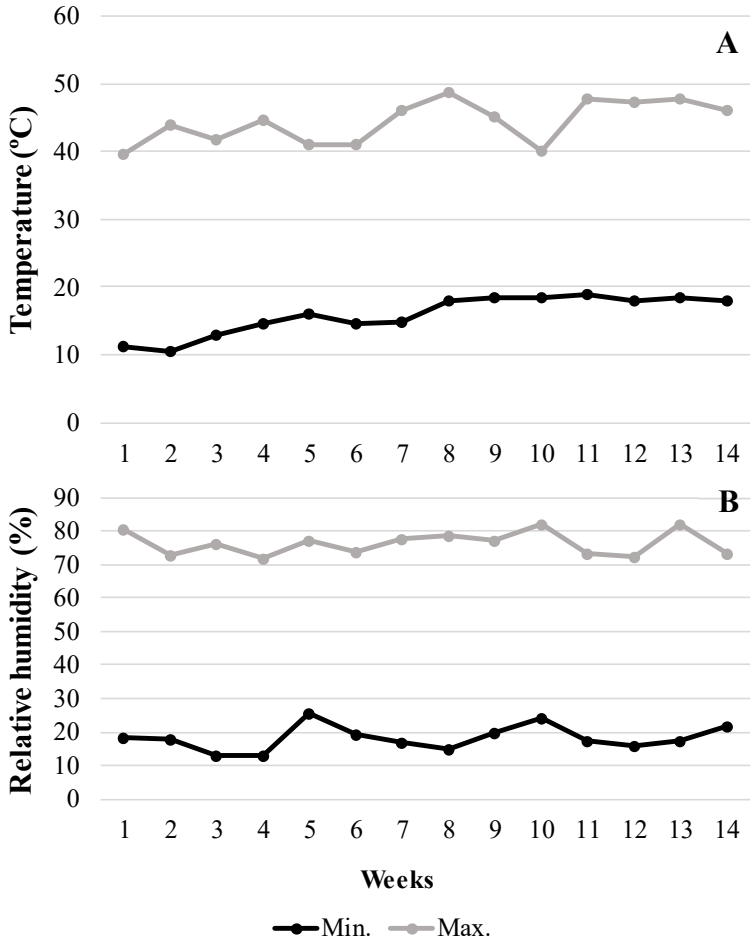

Fig. S1. Minimum and maximal temperature (**A**) and relative humidity (**B**) of the greenhouse during the period of cultivation of sea lavender plants.

Table S1. Nutrient profile (ammonia, NH_4^+; nitrates, NO_3^-; nitrites, NO_2^-; silica, $Si(OH)_4$; and phosphates, HPO_4^{2-}) of aquaculture wastewater during irrigation period (12 weeks). Values are expressed as µM.

Weeks	NH_4^+	NO_3^-	NO_2^-	$Si(OH)_4$	HPO_4^{2-}
1 – 4	0.18	0.05	0.11	5.55	0.21
5 – 8	0.28	0.01	0.15	0.43	0.14
9 – 12	38.9	1.64	0.91	12.3	1.04

CHAPTER 5

BIOLOGICAL ACTIVITIES AND CHEMICAL COMPOSITION OF *POLYGONUM MARITIMUM*

Rodrigues MJ, Custódio L, Lopes A, Oliveira M, Neng NR, Nogueira JMF, Martins A, Rauter AP, Varela J, Barreira L (2017). Unlocking the *in vitro* anti-inflammatory and antidiabetic potential of *Polygonum maritimum*. Phamaceutical Biology, 55, 1348-1357

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Unlocking the *in vitro* anti-inflammatory and antidiabetic potential of *Polygonum maritimum*

Maria João Rodrigues[a], Luísa Custódio[a], Andreia Lopes[a], Marta Oliveira[a], Nuno R. Neng[b], José M. F. Nogueira[b], Alice Martins[b], Amélia P. Rauter[b], João Varela[a] and Luísa Barreira[a]

[a]CCMAR, Centro de Ciências do Mar, Universidade do Algarve, Faro, Portugal; [b]Centro de Química e Bioquímica, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

ABSTRACT

Context: Several *Polygonum* species (Polygonaceae) are used in traditional medicine in Asia, Europe and Africa to treat inflammation and diabetes.

Objective: Evaluate the *in vitro* antioxidant, anti-inflammatory and antidiabetic potential of methanol and dichloromethane extracts of leaves and roots of the halophyte *Polygonum maritimum* L.

Material and methods: Antioxidant activity was determined (up to 1 mg/mL) as radical-scavenging activity (RSA) of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), copper (CCA) and iron (ICA) chelating activities and iron reducing power (FRAP). NO production was measured in lipopolysaccharide (LPS)-stimulated macrophages for 24 h at concentrations up to 100 μg/mL and antidiabetic potential was assessed by α-amylase and α-glucosidase inhibition (up to 10 mg/mL) assays. The phytochemical composition of the extracts was determined by gas chromatography-mass spectrometry (GC-MS).

Results: The methanol leaf extract had the highest activity against DPPH· (IC_{50} = 26 μg/mL) and $ABTS^{+}$· (IC_{50} = 140 μg/mL), FRAP (IC_{50} = 48 μg/mL) and CCA (IC_{50} = 770 μg/mL). Only the dichloromethane leaf extract (LDCM) showed anti-inflammatory activity (IC_{50} = 48 μg/mL). The methanol root (IC_{50} = 19 μg/mL) and leaf (IC_{50} = 29 μg/mL) extracts strongly inhibited baker's yeast α-glucosidase, but LDCM had higher rat's α-glucosidase inhibition (IC_{50} = 2527 μg/mL) than acarbose (IC_{50} = 4638 μg/mL). GC-MS analysis identified β-sitosterol, stigmasterol, 1-octacosanol and linolenic acid as possible molecules responsible for the observed bioactivities.

Conclusions: Our findings suggest *P. maritimum* as a source of high-value health promoting commodities for alleviating symptoms associated with oxidative and inflammatory diseases, including diabetes.

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Introduction

The genus *Polygonum* (Polygonaceae) includes more than 200 species worldwide, mainly in areas of temperate climate. Several *Polygonum* species are used in traditional medicine in China and Japan to treat health disorders such as dysentery, articular pain and inflammation (Takasaki et al. 2001; Kawai et al. 2006; Fan et al. 2011). Some species are also used in traditional medicine in Europe, Africa and Asia to treat diabetes (Soumyanath 2005; Bothon et al. 2013). In Europe, approximately 36 species of *Polygonum* can be found, including *P. maritimum* L., commonly known as sea knotgrass. Sea knotgrass is a perennial halophyte herb native from the sandy coasts of Europe, Mediterranean and Black Sea, Channel Islands, England and Belgium, occurring frequently throughout the Portuguese coast (Kilinc & Karaer 1995; Caçador et al. 2013). *Polygonum maritimum* has described antioxidant and antimicrobial activities (El-Haci et al. 2013), and contains bioactive molecules such as polygonocinol, (+)-8-hydroxycalamene, octacosyl, triacontyl ferulate, arylpropane, quercetin, quercitrin, (+)-catechin, and sitosterol (Kazantzoglou et al. 2009).

Diabetes is an emerging health problem in western societies affecting more than 300 million people worldwide and is expected to be the 7th cause of death by 2030 (Mathers & Loncar 2006; Danaei et al. 2011). Type 2 diabetes mellitus (T2DM) is mainly associated with genetics and lifestyle and encompasses more than 90% of all diabetes cases globally (Mozaffarian et al. 2009). The major characteristic of T2DM is high blood glucose level, which is caused by congenital or acquired deficiency in secretion of insulin combined with decreased responsiveness to this hormone (WHO 1999; Yarchoan & Arnold 2014). The inhibition of carbohydrate-hydrolyzing enzymes, namely α-amylase and α-glucosidase, is thus an important strategy to manage hyperglycaemia linked to T2DM by decreasing the postprandial raise in blood glucose levels (Kwon et al. 2007). Acarbose, miglitol and voglibose are clinically used compounds that target α-amylase and α-glucosidase; however, they present several side effects such as abdominal distension, flatulence and meteorism (Bischoff & Flower 1985). In this sense, there has been a growing effort to search for novel natural compounds with antidiabetic properties and reduced side effects (Kwon et al. 2007).

Hyperglycaemia found in T2DM patients may also induce metabolic disturbances leading to the development of oxidative stress and chronic inflammation states that contribute to diabetes-associated complications, namely, cardiovascular,

CONTACT Luísa Barreira lbarreir@ualg.pt CCMAR Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

urological, neurological, kidney and eyes disorders (American Diabetes Association 2010; Vikram et al. 2014). Oxidative stress coupled with reduced antioxidant defences enhances damage caused by free radicals, such as reactive oxygen species (ROS), and contributes to disease progression (Sabu & Kuttan 2002; Maritim et al. 2003). In this context, natural antioxidants can be useful in the prevention and/or management of oxidative stress-related disorders, including diabetes (Ruhe & McDonald 2001; Fardoun 2007). ROS also contributes to the production of pro-inflammatory cytokines and chemokines and to insulin resistance (Akash et al. 2013; Muriach et al. 2014). The role of oxidative stress and chronic inflammation in the progression of T2DM thus opens new avenues in the search for novel and combined therapies comprising the prevention of oxidative and inflammatory states (Akash et al. 2013).

As stated before, several *Polygonum* species are used in traditional medicine to treat inflammation and diabetes. However, to the best of our knowledge, there is no information regarding the anti-inflammatory and/or antidiabetic potential of the sea knotgrass. In this context, we report for the first time a comparative evaluation of the antioxidant and anti-inflammatory potential and inhibitory activity on key enzymes relevant for hyperglycaemia (α-amylase and α-glucosidase) of extracts of sea knotgrass leaves and roots. The phytochemical characterization of the extracts is also presented.

Material and methods

Chemicals, culture media and supplements

Sigma-Aldrich (Germany) supplied the 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, sodium nitrite, lipopolysaccharide (LPS) from *Escherichia coli*, sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NED) and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT). Folin-Ciocalteau (F-C) phenol reagent and phosphoric acid were purchased from Merck (Germany). Lonza (Belgium) provided Roswell Park Memorial Institute (RMPI) 1640 medium, fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin. Additional chemicals were acquired from VWR International (Belgium).

Sample collection

Whole plants of *P. maritimum* were hand collected in Ludo, South of Portugal, in June 2013. The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto from the National Museum of Natural History (University of Lisbon, Botanical Garden, Portugal) and a voucher specimen is kept in the herbarium of MarBiotech laboratory (MBH22). Plants were divided in roots and leaves, washed, oven dried for 3 days at 50 °C, powdered and stored at −20 °C.

Preparation of the extracts

Dried samples were separately extracted with methanol and dichloromethane (1:40, w/v), overnight at room temperature (RT), under stirring. The extracts were filtered (Whatman no. 4), and evaporated to dryness at 40 °C in a rotary evaporator under reduced pressure (BUCHI R-210, Flawil, Switzerland). The dried extracts were dissolved in the corresponding solvent at the concentration of 10 mg/mL to be used in the chemical characterization assays, or in dimethyl sulfoxide (DMSO) to be used in the bioactivity assays. All samples were stored at −20 °C until needed.

Gas chromatography and mass spectrometry (GC-MS) phytochemical analysis

The extracts (100 µL) were filtered (0.2 µm polytetrafluoroethylene membrane syringe filters), transferred to a glass vial and the solvent evaporated under a nitrogen stream. When dried, 50 µL of the derivatization reagent [*N*-methyl-N-(trimethylsilyl) trifluoroacetamide; MSTFA] was added. With the vial capped, the extracts were vortexed and heated for 20 min in a dry block heater at 40 °C (Pereira et al. 2012).

The GC/MS analyses were performed on an Agilent 6890 series gas chromatograph equipped with an Agilent 7683 automatic liquid sampler coupled to an Agilent 5973 N mass selective detector (Agilent Technologies, Little Falls, DE). A programed temperature vaporization injector with a septumless sampling head (Gerstel, Mullheim a/d Ruhr, Germany) and a baffled liner was used, operating in the solvent vent mode with compressed air for inlet cooling. Large volume injection was performed (vent time, 0.30 min; flow, 50 mL/min; pressure, 0 psi; purge, 60 mL/min at 2 min), for which the inlet temperature was programed from 60 °C (0.4 min) to 300 °C (3 min isothermal) at a rate of 60 °C/min and subsequently decreased to 200 °C (held until end) at a rate of 50 °C/min. The injection volume and speed were set at 10 µL and 100 µL/min, respectively. GC analysis was performed on a Zebron ZB-5 (30 m × 0.25 mm I.D., 0.25 µm df; Phenomenex, USA) capillary column (5% phenyl, 95% polydimethylsiloxane), using helium as carrier gas maintained in a constant inlet pressure mode of 7.81 psi. The oven temperature was programed from 100 °C (1 min) at 20 °C/min to 250 °C, then at 10 °C/min to 300 °C and hold for 20 min. The transfer line, ion source and quadrupole analyzer temperatures were maintained at 280 °C, 230 °C and 150 °C, respectively and a solvent delay of 4 min was selected. In the full-scan mode, electron ionization mass spectra in the range 35-550 Da were recorded at 70 eV with an ionization current of 34.6 µA. Data recording and instrument control were performed by MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies).

Radical-scavenging activity (RSA) on DPPH•

The RSA against DPPH was determined according to the method described by Custódio et al. (2015). Extracts (22 µL at concentrations ranging from 60 to 1000 µg/mL) were mixed with 200 µL of DPPH solution (120 µM, in methanol) in 96-well microplates and incubated in the dark for 30 min (RT). The absorbance was measured at 517 nm (Biotek Synergy 4) and results presented as half maximal inhibitory concentration (IC_{50}, µg/mL). Butylated hydroxytoluene (BHT) was used as a positive control.

RSA on ABTS•+

The RSA against ABTS radical was evaluated by the method described previously (Rodrigues et al. 2015). 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). Extracts (10 µL at concentrations from 60 to 1000 µg/mL) were mixed in 96-well microplates with 190 µL of $ABTS^{\bullet+}$ solution. After 6 min

of incubation the absorbance was measured at 734 nm (Biotek Synergy 4). Results were expressed IC_{50} values (μg/mL). BHT was used as positive control.

RSA on nitric oxide ($NO^{\bullet}$)

The $NO^{\bullet}$ scavenging activity was evaluated according to Rodrigues et al. (2015) on extracts at concentrations between 60 and 1000 μg/mL. Samples (50 μL) were mixed with 50 μL of 10 mM sodium nitroprusside in phosphate buffer (PBS) and incubated for 90 min at RT. After, 50 μL of Griess reagent (1% of sulfanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO_3) were added. The absorbance was read at 546 nm, and results were expressed as IC_{50} values (μg/mL). N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as standard.

Copper (Cu^{2+}) chelating activity (CCA)

The CCA was assessed using pyrocatechol violet as described previously (Rodrigues et al. 2015). The extracts (30 μL) were applied at concentrations from 60 to 1000 μg/mL and mixed 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 $CuSO_4{\cdot}5H_2O$ (50 μg/mL, in water). The change in the colour of the solution was measured at 632 nm using a microplate reader (Biotek Synergy 4). Results were expressed as IC_{50} values (μg/mL). Ethylenediamine tetraacetic acid (EDTA) was used as a positive control.

Iron (Fe^{2+}) chelating activity (ICA)

The ICA chelating activity was determined by measuring the formation of the Fe^{2+} ferrozine complex (Megías et al. 2009), according to Rodrigues et al. (2015). Extracts (30 μL at concentrations between 60 and 1000 μg/mL) were mixed in 96-well microplates with 200 μL of dH_2O and 30 μL of a $FeCl_2$ solution (0.1 mg/mL in water). After 30 min, 12.5 μL of ferrozine solution (40 mM in water) was added. The change in colour was measured in a microplate reader (Biotek Synergy 4) at 562 nm, and results were expressed as IC_{50} values (μg/mL). EDTA was used as standard.

Ferric reducing antioxidant power (FRAP) assay

The ability of the extracts to reduce Fe^{3+} was assayed by the method described by Megías et al. (2009). Extracts (50 μL) were tested at concentrations ranging from 60 to 1000 μg/mL and mixed with distilled water (50 μL) and 1% potassium ferricyanide (50 μL). After an incubation of 20 min at 50 °C, 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. Increased absorbance of the reaction mixture indicates increased reducing power. Antioxidant activity was calculated relatively to the positive control (BHT; 1000 μg/mL), and expressed as IC_{50} values (μg/mL).

Cell culture and cell viability

RAW 264.7 macrophages were maintained in RPMI culture medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL)/streptomycin (50 μg/mL), and were maintained at 37 °C in humidified atmosphere with 5% CO_2. Exponentially growing cells were plated in 96-well tissue plates at a concentration of 1×10^4 cells/well and incubated for 24 h. Extracts were then applied at different concentrations (3 to 100 μg/mL) for 24 h. Control cells were treated with DMSO at the highest concentration used in test wells (0.5%), and cell viability was determined by the MTT colorimetric assay (Mosmann 1983). Briefly, 2 h prior to the end of the incubation period 20 μL of MTT (5 mg/mL in PBS) were added to each well and further incubated at 37 °C. Then, 150 μL of DMSO was added to each well in order to dissolve the formazan crystals and absorbance was measured at 590 nm (Biotek Synergy 4).

Measurement of NO production

The NO production was evaluated using RAW 264.7 macrophages as described by Rodrigues et al. (2014). Cells were plated at 2.5×10^5 cells/mL in 96-well tissue plates and allowed to adhere overnight. Afterwards, they were treated with nontoxic 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, for 24 h (Nishishiro et al. 2005). NO production was assessed using the Griess assay (Miranda et al. 2001). A calibration curve was prepared with different concentrations of sodium nitrite (1.5–100 μM). Results were expressed as percentage (%) of NO production, relative to a control containing DMSO (0.5%, v/v), and as IC_{50} values (μg/mL).

α-Amylase inhibitory activity

The α-amylase inhibitory activity was determined by the method described by Xiao et al. (2006). Samples (40 μL at concentrations ranging from 1000 to 10,000 μg/mL) were mixed in 96-well microplates with 40 μL of amylase solution (100 U/mL in 0.1 M sodium phosphate buffer, pH 7.0) and 40 μL of 0.1% starch solution (diluted in the previous buffer). After 10 min at 37 °C, 20 μL of 1 M hydrochloric acid (HCl) and 100 μL of iodide solution (5 mM iodine (I_2) + 5 mM potassium iodide (KI), in distilled water) were added and the absorbance was measured at 580 nm. Results were expressed as IC_{50} values (μg/mL). Acarbose was used as the standard at concentrations between 250 and 10,000 μg/mL.

Baker's yeast α-glucosidase inhibitory activity

Microbial (*Saccharomyces cerevisiae*) α-glucosidase inhibitory activity was determined according to the method described by Custódio et al. (2015). Samples (50 μL at concentrations ranging from 20 and 1000 μg/mL) were mixed with 100 μL of enzyme solution (1.0 U/mL, in 0.1 M sodium phosphate buffer, pH 7.0), and incubated for 10 min at 25 °C. Then, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (NGP; diluted in the previous buffer) were added and incubated more 5 min at 25 °C. The absorbance was recorded at 405 nm using a microplate reader (Biotek Synergy 4) and results were expressed as IC_{50} values (μg/mL). Acarbose was used as positive control at concentrations from 250 to 10,000 μg/mL.

Rat's intestinal α-glucosidase inhibitory activity

Rat's intestinal acetone powder was used as a crude enzyme extract as an example of enzyme of mammalian origin

(Kwon et al. 2007). Rat's intestinal acetone powder (250 mg) were mixed with 10 mL of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 5000 × *g* for 20 min at 4 °C. The supernatant (100 µL) was mixed with the extracts (50 µL at concentrations between 500 and 10,000 µg/mL), and incubated for 10 min at 25 °C. Then, 50 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (NGP; diluted in the previous buffer) was added and the mixture was incubated for 30 min at 37 °C. The absorbance was read at 405 nm using a microplate reader (Biotek Synergy 4), and results were expressed as IC_{50} values (µg/mL). Acarbose was used as positive control at concentrations from 250 to 10,000 µg/mL.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM), and experiments were carried out at least in triplicate. Significant differences were assessed by analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test, or by Kruskal–Wallis test when parametricity of data did not prevail. SPSS statistical package for Windows (release 15.0, SPSS Inc.) was used. The IC_{50} values were calculated by sigmoidal fitting of the data in the GraphPad Prism v. 5.0 software.

Results

Phytochemical analysis

In order to determine their chemical composition, extracts were analyzed by GC-MS (Figure 1 and Table 1). This analysis was able to identify a large number of compounds detected in the methanol extracts (70–75%). However, a large percentage of compounds detected in the dichloromethane extracts could not be identified (45–48%). In total, 51 compounds were identified belonging to different classes of biochemicals: alkanes and alkenes (AA), fatty acids (FA), phenolic compounds (PC), acylglycerols (GLY), saccharides (SAC), alcohols (ALC), phytosterols (PS) and minor groups (MG).

With similar contents, fatty acids (24.3%) and acylglycerols (22.8%) were the most represented categories in the methanol leaf extract (LM), in which 2-monostearin (**35**) was the major compound (15.4%). Palmitic acid (**18**), linolenic acid (**23**), 1-monopalmitin (**34**), glycerol (**43**), and β-sitosterol (**47**) were also detected at abundances higher than 5%. Acylglycerols were the most abundant constituents of the LM extract (34.4%), with 1-monostearin (**31**) being the most representative compound (24.0%), while 1-monopalmitin (**34**) and oleamide (**51**)

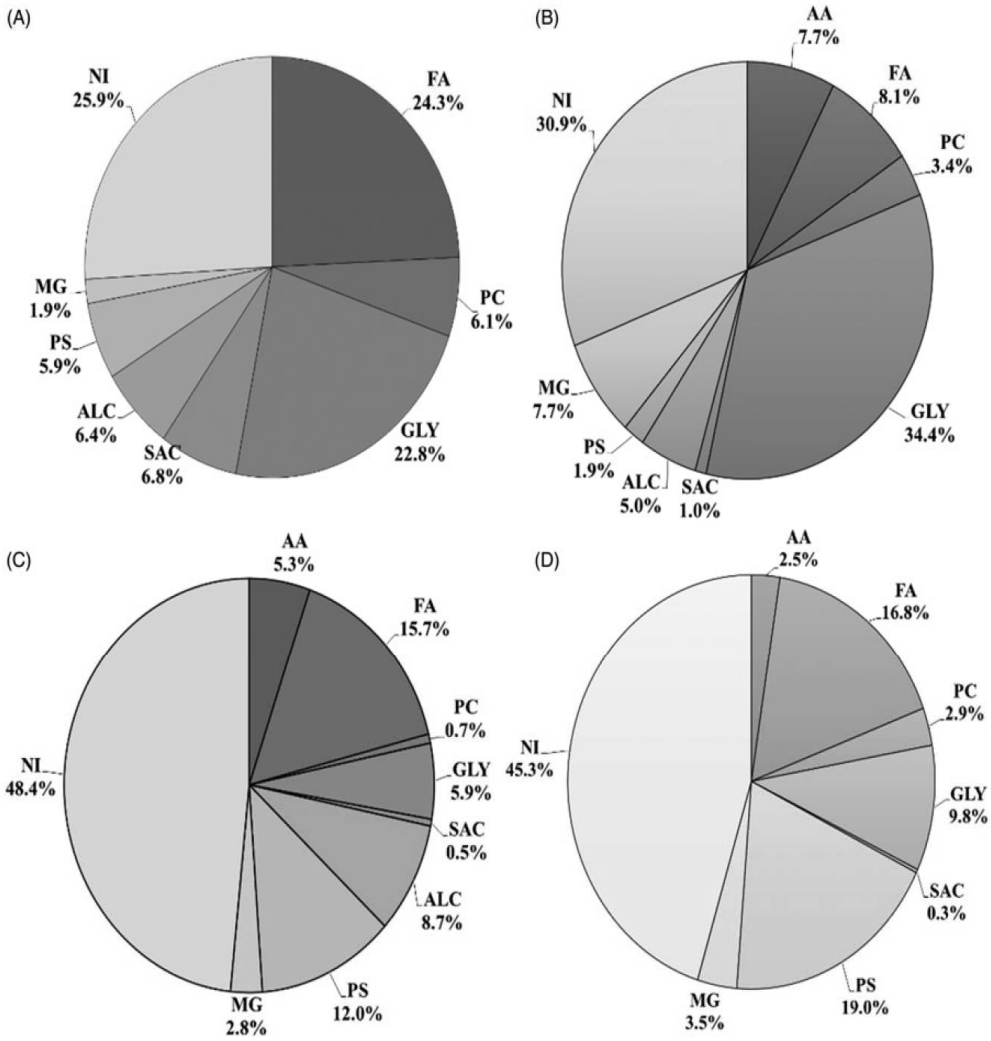

Figure 1. Main chemical compound classes identified by GC/MS in the dichloromethane and methanol extracts of roots and leaves of *P. maritimum*. (A) Methanol leaf extract; (B) Methanol root extract; (C) Dichloromethane leaf extract; and (D) Dichloromethane root extract. AA: alkanes and alkenes; FA: fatty acids; PC: phenolic compounds; GLY: acylglycerols; SAC: saccharides; ALC: alcohols; PS: phytosterols; MG: minor groups; NI: non-identified compounds.

Table 1. Phytochemical composition of the methanol and dichloromethane extracts of *P. maritimum* leaves and roots, determined by GC/MS analysis.

				Relative abundance (%)			
				Methanol		Dichloromethane	
ID	RT (min)	Compound	Molecular formula	Leaves	Roots	Leaves	Roots
Alkanes/Alkenes							
1	5.61	2,6,11-Trimethyl dodecane	$C_{15}H_{32}$		0.67		
2	5.63	Phytane	$C_{20}H_{42}$			0.22	0.21
3	5.66	2,6,10,15-Tetramethyl heptadecane	$C_{17}H_{36}$		0.72		
4	6.75	Tetradecane	$C_{14}H_{30}$		0.97	0.32	0.40
5	8.55	Cetane	$C_{16}H_{34}$			0.16	0.18
6	8.63	2-Methyl octadecane	$C_{19}H_{40}$		0.78	0.26	
7	9.78	Octacosane	$C_{28}H_{58}$		0.77	0.37	0.70
8	9.90	3,11-Dimethyl nonacosane	$C_{31}H_{64}$		1.10		
9	11.57	Eicosane	$C_{20}H_{42}$		0.90		
10	12.39	Docosane	$C_{22}H_{46}$		1.78		1.01
11	12.40	Tricosane	$C_{23}H_{48}$			0.73	
12	14.10	Pentacosane	$C_{25}H_{52}$			0.59	
13	16.89	1-Nonadecene	$C_{19}H_{38}$			0.87	
14	17.75	Nonacosane	$C_{29}H_{60}$			1.80	
Fatty acids							
15	5.75	Pelargonic acid	$C_{12}H_{18}O_2$	0.28			0.17
16	7.72	Lauric acid	$C_{12}H_{24}O_2$	1.51		0.25	0.23
17	8.92	Myristic acid	$C_{14}H_{28}O_2$	1.22		1.72	0.65
18	10.28	Palmitic acid	$C_{16}H_{32}O_2$	6.40	1.46	4.49	6.53
19	11.08	Linoleic acid	$C_{18}H_{32}O_2$	1.79	2.32	0.35	
20	11.18	Margaric acid	$C_{17}H_{34}O_2$			0.14	0.20
21	11.75	Oleic acid	$C_{18}H_{34}O_2$	4.13		2.32	3.21
22	11.85	Linolenic acid	$C_{18}H_{30}O_2$			0.95	
23	11.94	Stearic acid	$C_{18}H_{36}O_2$	6.71	4.27	1.83	1.38
24	13.59	Arachidic acid	$C_{20}H_{40}O_2$	1.71		1.05	
25	15.32	Behenic acid	$C_{22}H_{44}O_2$			1.35	2.64
26	17.10	Lignoceric acid	$C_{24}H_{48}O_2$	0.53		1.29	1.78
Phenols							
27	5.09	Benzoic acid	$C_7H_7O_2$	3.24	0.51		
28	6.94	Butylated hydroxytoluene	$C_{15}H_{24}O$	0.99	1.31	0.17	0.27
29	7.12	Vanillin	$C_8H_8O_3$				0.68
30	7.70	Phloroglucinol	$C_6H_6O_3$		0.92		
31	9.63	Methyl 3-(3,4-di-tert-butyl-4-hydroxyphenyl)propionate	$C_{18}H_{28}O_3$	0.68	0.66		
32	9.69	Gallic acid	$C_7H_6O_5$	1.15		0.54	1.97
Acylglycerols							
33	14.63	2-Monopalmitin	$C_{19}H_{38}O_4$				1.28
34	14.89	1-Monopalmitin	$C_{19}H_{38}O_4$	5.89	10.3	2.20	3.05
35	16.14	2-Monostearin	$C_{19}H_{38}O_4$	15.4		0.34	
36	16.27	1-Monoolein	$C_{21}H_{40}O_4$	0.35		0.47	
37	16.59	1-Monostearin	$C_{19}H_{38}O_4$	1.08	24.1	2.87	5.44
Saccharides							
38	8.69	D-Fructose	$C_6H_7O_6$	4.40	0.97	0.39	0.27
39	9.02	β-D-Glucofuranose	$C_6H_{12}O_6$	0.85			
40	9.34	β-D-Glucopyranose	$C_6H_{12}O_6$	1.05		0.14	
41	9.41	α-D-Glucopyranose	$C_6H_{12}O_6$	0.45			
Alcohols							
42	4.12	Butane-1,3-diol	$C_4H_{10}O_2$	0.62			
43	5.18	Glycerol	$C_3H_8O_3$	5.20	5.02		
44	11.35	Phytol	$C_{20}H_{40}O$	0.62			
45	20.91	1-Octacosanol	$C_{28}H_{58}O$			8.72	
Phytosterols							
46	24.51	Stigmasterol	$C_{29}H_{48}O$			1.37	
47	25.88	β-Sitosterol	$C_{29}H_{50}O$	5.90	1.88	10.6	19.0
Minor groups							
48	5.25	Phosphoric acid	H_3PO_4	0.62	0.55	0.46	0.34
49	7.44	Diethyl phthalate	$C_{12}H_{14}O_4$	0.35			
50	7.78	Thymine	$C_5H_6N_2O_2$		0.46		
51	13.11	Oleamide	$C_{18}H_{35}NO$	0.89	6.66	2.30	3.13

represented 10.3 and 6.66% of the total identified components, respectively. In the dichloromethane leaf extract (LDCM), fatty acids represented 15.7% and phytosterols (PS) 12.0% of the total identified compounds. β-Sitosterol (**47**; 10.5%) and 1-octacosanol (**45**; 8.7%) were the major components identified. Similarly, the dichloromethane root extract (RDCM) had the highest content in phytosterols (PS; 19.0%) and fatty acids (FA; 16.7%), in which β-sitosterol (**47**, 19.0%) was the main component, followed by palmitic acid (**18**) and 1-monostearin (**37**) at 6.5 and 5.4%, respectively.

Antioxidant activity

The *P. maritimum* LM and methanol root (RM) extracts showed high RSA for both DPPH and ABTS radicals, coupled with a

Table 2. Radical scavenging activity (RSA) on DPPH, ABTS and NO radicals, metal chelating activity on iron (ICA) and copper (CCA) and ferric reducing activity (FRAP) activity of methanol (MeOH) and dichloromethane (DCM) extracts of roots and leaves of *P. maritimum*.

		RSA			Metal chelation/reduction		
Extract/Standard	Plant organ	DPPH	ABTS	NO	CCA	ICA	FRAP
MeOH	Leaves	26 ± 0.7^{a}	140 ± 6^{a}	n.a.	290 ± 10^{b}	n.a.	48 ± 1.4^{a}
	Roots	27 ± 0.0^{a}	192 ± 5^{b}	n.a.	446 ± 8^{c}	n.a.	64 ± 3.8^{a}
DCM	Leaves	n.a.	n.a.	n.a.	n.a.	n.a.	798 ± 36^{b}
	Roots	n.a.	n.a.	n.a.	n.a.	n.a.	770 ± 53^{b}
BHT*	–	110 ± 10^{b}	140 ± 10^{a}	–	–	–	–
L-NAME*	–	–	–	2500 ± 10	–	–	–
EDTA*	–	–	–	–	170 ± 10^{a}	55.9 ± 3.7	–

Results are expressed as IC_{50} values (μg/mL). *Positive control; n.a.: not active. Values are means ± SEM of three separate experiments performed in triplicate ($n = 9$). In the same column, means labelled with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

strong capacity for reducing iron and chelating copper (Table 2). In the DPPH assay, the methanol extracts of both organs had similar IC_{50} values (roots: 27 μg/mL; leaves: 26 μg/mL), which were significantly lower than the one obtained with the positive control (BHT, 110 μg/mL; Table 2). Regarding the ABTS radical, both the RM and LM extracts had IC_{50} of 192 and 140 μg/mL, respectively, which were similar to that of BHT (140 μg/mL; Table 2). The RM and LM had also high ability to reduce iron with IC_{50} values of 64 and 48 μg/mL, respectively (Table 2). A moderate iron reduction was obtained upon addition of the RDCM ($IC_{50} = 770$ μg/mL) and LDCM ($IC_{50} = 798$ μg/mL) extracts. However, none of the extracts had the capacity for scavenging the NO radical or for chelating iron (Table 2).

Anti-inflammatory activity

To assess the *in vitro* anti-inflammatory activity, the effect of applying nontoxic concentrations of the extracts (i.e. yielding cell viability >80%) on the NO production by LPS-stimulated RAW 264.7 macrophage cells was determined. A significant reduction in cell viability was observed upon applying the LM extract at a concentration of 100 μg/mL to the cells. Loss of viability was also observed with the RDCM extract at 50 and 100 μg/mL (data not shown). Therefore, these concentrations were not used in the anti-inflammatory activity assessment.

Exposure of RAW 264.7 cells to LPS at 100 ng/mL increased the nitrite concentration in the culture medium from a basal level of approximately 0.3 μM to around 13 μM (data not shown). This increase was significantly reduced in a dose-dependent manner by the treatment with LDCM, at concentrations ranging from 25 to 100 μg/mL, showing that the latter had an activity similar to the positive control (L-NAME) at the same concentration (Figure 2). However, this extract had an IC_{50} value of 48 μg/mL, higher than that of L-NAME (29.1 μg/mL; data not shown). Interestingly, incubating this cell line with the remaining extracts resulted in an increase in NO production when compared to the control (Figure 2). In particular, incubation with LM and RM extracts resulted in the most significant increases in NO production: 144% at the concentration of 50 μg/mL and 139% at 25 μg/mL, respectively.

Antidiabetic activity

The inhibitory potential of extracts from *P. maritimum* was evaluated against α-amylase and baker's yeast and rat's α-glucosidases (Table 3). The methanol extracts had the highest capacity to inhibit the baker's yeast α-glucosidase with IC_{50} values of 19 and 29 μg/mL for roots and leaves, respectively, which were significantly lower than that of acarbose ($IC_{50} = 3144$ μg/mL). Despite the fact that methanol extracts had no capacity to inhibit rat's α-glucosidase, LDCM had the highest rat's α-glucosidase inhibitory activity ($IC_{50} = 2527$ μg/mL), which was a significantly lower IC_{50} value than that of acarbose (4638 μg/mL; $p < 0.05$).

Discussion

In order to confirm the use of *Polygonum* species in traditional medicine in the treatment of inflammation and diabetes, we assessed for the first time the phytochemical composition of *P. maritimum* and its *in vitro* anti-inflammatory and antidiabetic potential.

GC-MS analysis detected alkanes, alkenes and fatty acids as the major categories of compounds in *P. maritimum* extracts. These are lipophilic compounds widely distributed in plants as constituents of plant waxes and have been described in the halophilic Mediterranean *Suaeda vera* Forssk. ex J.F.Gmel. (Amaranthaceae), *Sarcocornia fruticosa* (L.) A.J.Scott (Amaranthaceae), and *Halimione portulacoides* (L.) Aellen (Amaranthaceae) (Grossi & Raphel 2003). Waxes usually have a protective role, for example, against microbial infections and avoiding excessive water losses and acute osmotic stress that halophilic species suffer while growing in drenched soils (Müller & Riederer 2005; Huang et al. 2011). Moreover, they are common constituents of essential oils of halophyte species, such as *Suaeda fructicosa* and *Limonium echioides* (L.) Mill. (Plumbaginaceae) (Saïdana et al. 2008). From the compounds detected in this study, only β-sitosterol (**47**) had been previously identified in *P. maritimum* also in dichloromethane extracts. Phytol (**44**), palmitic (**18**) and lauric (**16**) acids were also previously detected as components of essential oils of *Polygonum* species, namely *P. hydropiper* L. and *P. minus* Huds. (Miyazawa & Tamura 2007; Baharum et al. 2010).

Our findings showed that *P. maritimum* has a strong antioxidant activity comparable to that reported by other authors for similar extracts made from aerial parts of the same species (El-Haci et al. 2013) and *P. sachalinensis* F.Schmidt and *P. cuspidatum* Siebold & Zucc. (Pan et al. 2007; Fan et al. 2011). Moreover, IC_{50} values obtained with the RM and LM were significantly lower than or similar to the one obtained with the positive control (BHT) for DPPH and ABTS assays, respectively. The significantly higher antioxidant activity of these extracts can be related to the presence of some of the compounds identified in these extracts, namely, the phenolic compounds benzoic acid, BHT, vanillin and phloroglucinol, since these compounds are

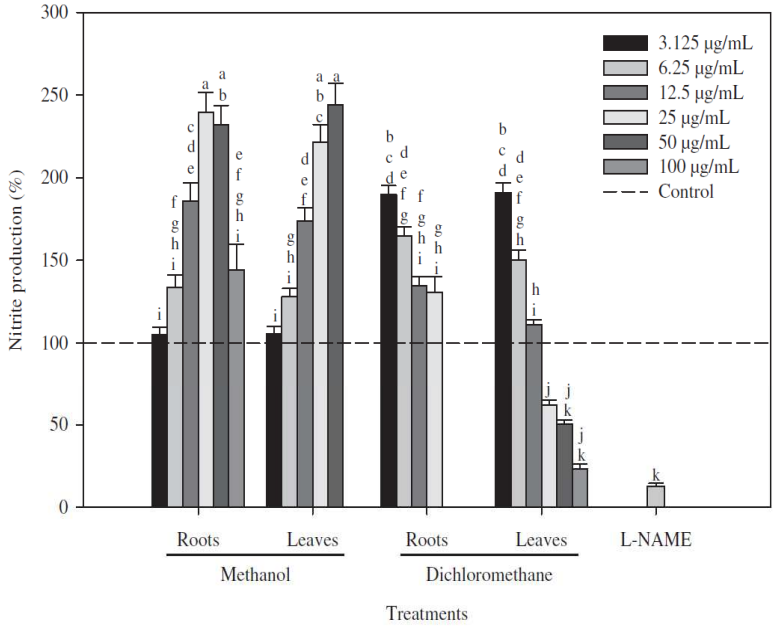

Figure 2. Effect of the application of dichloromethane and methanol extracts of roots and leaves of *P. maritimum* on NO production (%) by LPS-stimulated macrophages. Control cells were treated with culture medium supplemented with 0.5% DMSO and 100 ng/mL of LPS. L-NAME (positive control) was applied at the concentration of 100 µg/mL. Solid and errors bars represent the average and SEM, respectively ($n = 9$). Bars followed by different letters are significantly different according to the Duncan's multiple ranges test ($p < 0.05$).

Table 3. Inhibitory activity of dichloromethane and methanol extracts of roots and leaves of *P. maritimum* on α-amylase, baker's yeast α -glucosidase and rat's intestinal α-glucosidase.

Extract/Standard	Organ	α-Amylase	Yeast α-glucosidase	Rat α-glucosidase
Methanol	Leaves	n.a.	29 ± 0.7^{a}	n.a.
	Roots	n.a.	19 ± 0.5^{a}	n.a.
Dichloromethane	Leaves	n.a.	585 ± 27^{b}	2527 ± 37
	Roots	n.a.	626 ± 14^{b}	>2500
Acarbose*		7797 ± 98	3144 ± 132^{c}	4638 ± 438

Results are expressed as IC_{50} values (µg/mL). *Positive control; n.a.: not active. Values are means ± SEM of three separate experiments performed in triplicate ($n = 9$). In the same column, means labelled with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

well described as strong antioxidants *in vitro* (Foti 2007; Dai & Mumper 2010). Phytol and linolenic acid have also been described as potent *in vitro* and *in vivo* antioxidants due to their hydroxyl group (Richard et al. 2008; Santos et al. 2013). Thus, their presence in the LM could also contribute to the high RSA and ICA of this extract. Interestingly, no significant differences were observed between the antioxidant activity of leaves and roots as opposed to that reported for other halophytes species, such as *Mesembryanthemum edule* L. (Aizoaceae), *Limoniastrum monopetalum* (L.) Boiss. (Plumbaginaceae), *Salsola kali* L. (Chenopodiaceae), *Tamarix gallica* L. (Tamaricaceae) and *Limonium algarvense* Erben (Plumbaginaceae) (Ksouri et al. 2008; Falleh et al. 2012; Trabelsi et al. 2012; Rodrigues et al. 2015). This can be explained by the similar phytochemical profile of the methanol extracts of both organs (Figure 1 and Table 1), suggesting that the bioactive compound(s) are not organ-specific.

LPS is a cell wall endotoxin produced by Gram-negative bacteria that activates macrophages to produce inflammatory mediators such as NO (Martich et al. 1993), a radical associated with chronic inflammation (Kubes 2000; Joo et al. 2014). In this context, a decrease in NO production is used as an indicator of the potential for the extracts to reduce an inflammatory response (Joo et al. 2014; Rodrigues et al. 2014). On the other hand, an increase in NO production can indicate an immunostimulatory effect of the extracts, which is important in macrophage defence and protection against infection (Wink et al. 2011).

The anti-inflammatory effect of *P. maritimum* leaves may be attributed to the presence of compound(s) with potential anti-inflammatory properties, most likely β-sitosterol, stigmasterol, 1-octacosanol, oleamide as well as linolenic and oleic acids (Table 1). For instance, β-sitosterol, one of the major constituents of this extract, has anti-inflammatory properties through TNF-α inhibition (Loizou et al. 2010), and so do 1-octacosanol and oleic acid (Vassiliou et al. 2009; de Oliveira et al. 2012). In turn, linolenic acid and oleamide are able to reduce NO production and inducible nitric oxide synthase (iNOS) gene expression, through the inhibition of the NF-κB pathway (Ren & Chung 2007; Oh et al. 2010). Stigmasterol was also reported to inhibit the production of pro-inflammatory mediators associated with the same pathway (Gabay et al. 2010). The combination of all these compounds in the LDCM of *P. maritimum* are probably contributing to its NO inhibitory capacity, since they were solely identified in this extract, or in higher quantities (Figure 2). These findings are in accordance with several reports on the anti-inflammatory properties of *Polygonum* species (*P. lapathifolium L.*, *P. cuspidatum* and *P. perfoliatum* L. (Takasaki et al. 2001; Kim et al. 2007; Fan et al. 2011; Lei et al. 2015).

NO has an important role in the immune system modulation, being one of the macrophage-mediated primary responses against pathogens such as fungi, helminthes, protozoa and bacteria (Wink et al. 2011). In this sense, the increased NO production suggests that both the LM and RM from *P. maritimum* may have immunostimulatory properties (Wink et al. 2011) most likely due to the presence of specific molecules in these extracts, namely, saturated fatty acids (stearic and palmitic acids), which are known inducers of the production of pro-inflammatory cytokines in macrophages (Valdearcos et al. 2012; Miao et al. 2015). In fact, immunostimulatory properties are reported in

P. multiflorum, P. minus and *P. cuspidatum* (Chen et al. 2012; Veerasamy et al. 2014; Chueh et al. 2015).

Compounds with the capacity to inhibit carbohydrate-hydrolyzing enzymes like α-amylase and α-glucosidase can delay the digestion of carbohydrates, decreasing the postprandial increase of blood glucose level after a mixed carbohydrate meal, and therefore can be an important strategy in managing hyperglycaemia linked to T2DM (Krentz & Bailey 2005; Kwon et al. 2007; Bhandari & Ansari 2008).

It is noteworthy that methanol extracts were approximately 100-fold more active towards baker's yeast α-glucosidase than acarbose and that the LDCM had 2-times more ability to inhibit rat's α-glucosidase. High IC_{50} values for acarbose against this enzyme have also been reported by other authors (4823 µg/mL) (Gao et al. 2013). However, the methanol extracts had no capacity to inhibit rat́s α-glucosidase, which is a common feature of molecules with inhibitory capacity on α-glucosidase from microbial origin (Oki et al. 1999; Shai et al. 2011). Furthermore, a few *in vitro* studies have discussed the low capacity of acarbose to inhibit mammalian α-glucosidase compared to crude extracts, including aqueous ethanol extracts from *P. senegalensis* (Meisn.) Soják (Polygonaceae), which is used in folk medicine to treat T2DM (Shinde et al. 2008; Bothon et al. 2013). Those differences are usually attributed to additive or synergistic interactions of the compounds present in the extracts, resulting in a higher capacity to inhibit the mammalian α-glucosidase (Adisakwattana et al. 2012). The higher activity of the LDCM can be related with the presence of some particular compounds. For example, β-sitosterol was the main compound identified in this extract (Table 1) and was previously reported to possess strong hypoglycaemic activity through α-glucosidase inhibition (Ortiz-Andrade et al. 2007). The same properties were described for stigmasterol and linolenic acid, present only in this extract, as well as for oleic acid (Ortiz-Andrade et al. 2007; Lean Teik et al. 2013; Su et al. 2013). In fact, Su et al. (2013) reported that linolenic and oleic acids were more active than acarbose. Although none of the extracts achieved 50% of inhibitory activity in the α-amylase assay, our data suggest that *P. maritimum* may have potential as a source of antidiabetic molecules. This is in accordance with the antidiabetic activity found in several *Polygonum* species, such as *P. aviculare* L. (Polygonaceae), *P. cuspidatum, P. multiflorum* and *P. senegalensis* (Soumyanath 2005; Bothon et al. 2013).

Conclusions

The present study highlights for the first time the potential of the halophyte *P. maritimum* as a source of compounds with antioxidant, anti-inflammatory and antidiabetic activities. The methanol extracts had the highest antioxidant capacity, possibly due to the presence of benzoic acid, BHT, phloroglucinol, phytol and linolenic acid. The dichloromethane extracts from *P. maritimum* leaves had significant anti-inflammatory activity, most likely related to its main constituents identified as β-sitosterol, stigmasterol, 1-octacosanol, oleamide, linolenic and oleic acids. Moreover, its high α-glucosidase inhibitory activity may be related to the presence of β-sitosterol, stigmasterol, linolenic and oleic acid. Overall, our results indicate that *P. maritimum* extracts are endowed with compounds with potential to be used as a combined strategy to manage T2DM due to its anti-inflammatory, antioxidant and α-glucosidase inhibitory properties. These results could be the starting points to further explore *P. maritimum*, especially its leaves, as a source of value-added bioactive natural products. Nonetheless, isolation and identification of the molecules(s) responsible for the detected biological activities is already being pursued.

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Disclosure statement

The authors report that they have no conflicts of interest.

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ORCID

Maria João Rodrigues http://orcid.org/0000-0001-8732-710X
Luísa Custódio http://orcid.org/0000-0003-4338-7703
Marta Oliveira http://orcid.org/0000-0001-6793-8026
Nuno R. Neng http://orcid.org/0000-0001-9879-1565
José M. F. Nogueira http://orcid.org/0000-0001-5785-6780
Alice Martins http://orcid.org/0000-0002-0542-2662
Amélia P. Rauter http://orcid.org/0000-0003-3790-7952
João Varela http://orcid.org/0000-0003-3101-693X
Luísa Barreira http://orcid.org/0000-0002-4077-855X

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Research paper

In vitro and *in silico* approaches to appraise *Polygonum maritimum* L. as a source of innovative products with anti-ageing potential

Maria João Rodrigues[a], Sylwester Slusarczyk[b], Łukasz Pecio[d], Adam Matkowski[b], Ramin Ekhteiari Salmas[c], Serdar Durdagi[c], Catarina Pereira[a], João Varela[a], Luísa Barreira[a], Luísa Custódio[a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Wroclaw, Poland
[c] Computational Biology and Molecular Simulations Laboratory, Department of Biophysics, School of Medicine, Bahcesehir University, Istanbul, Turkey
[d] Department of Crop Biochemistry, IUNG – Institute of Soil Science and Plant Cultivation, Pulawy, Poland

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ABSTRACT

Different *Polygonum* species have *in vitro* neuroprotective properties and are traditionally used for their anti-ageing benefits. In this context this work explored for the first time *P. maritimum* (sea knotgrass) as a potential source of natural products with industrial application as cognitive enhancers with anti-ageing potential. For that purpose methanol and dichloromethane extracts were prepared from leaves and roots of that species and evaluated for *in vitro* neuroprotective effects through the inhibition on acetyl- (AChE) and butyrylcholinesterase (BuChE), protection against hydrogen peroxide (H_2O_2)-induced cytoxicity on neuroblastoma cells and lipopolysaccharide (LPS)-induced neuroinflammation on microglia cells. The extracts were chemically characterized by ultra-high-pressure liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) and docking studies were performed on the identified compounds. Methanol extracts had the highest activity in AChE (leaves: IC_{50} = 0.27 mg/mL; roots: IC_{50} = 0.17 mg/mL) and BuChE (leaves: IC_{50} = 0.62 mg/mL; roots: IC_{50} = 0.61 mg/mL) inhibition, as well as reduction of nitric oxide (NO) production in LPS-treated microglia (leaves: IC_{50} = 4.17 µg/mL; roots: IC_{50} = 9.95 µg/mL). Methanol extracts prevented oxidative stress-induced cytotoxicity in SH-SY5Y cells when applied simultaneously with H_2O_2, whereas cells pre-treated with the dichloromethane extracts had increased viability. Fifteen flavonoids were identified and showed favorable binding energies to AChE and BuChE binding pockets. These data suggests *P. maritimum* as a promising source of natural products and/or molecules with cognitive enhancement and anti-ageing properties.

1. Introduction

Plants are outstanding sources of raw materials to be used in several industrial segments including those related with dietary supplements and food additives for human health improvement (Mocan et al., 2016; Zengin, 2016; Yerlikaya et al., 2017; Zengin et al., 2017). Halophytes in particular have a high but insufficiently explored biotechnological potential due to their unique chemical characteristics.

Halophytes are a group of approximately 2600 species of specialized plants which can, by definition, withstand, survive and reproduce at salt concentrations of 200 mM or higher (Koyro and Huchzermeyer, 2004; Flowers and Colmer, 2008). Halophytes are typically found in salt deserts and marine coastal areas such as beaches and salt marshes. In order to withstand the abiotic stresses that characterize those habitats, such as salinity and drought, these plants developed specific physiological and biochemical adaptations, as for example antioxidant enzymatic defense mechanisms and synthesis and accumulation of different metabolites, including phenolic compounds and alkaloids (Ksouri et al., 2012). Such compounds have not only protective properties for the plant, but also display biological properties relevant for human health improvement, as for example antioxidant, anti-inflammatory and neuroprotective (Flowers et al., 2010; Ksouri et al., 2012), which can explain their traditional medicinal uses and high biotechnological potential. *Polygonum maritimum* (sea knotgrass) is an obligatory halophyte that grows in the sandy coasts of Europe, Mediterranean and Black Sea regions, Channel Islands, England and Belgium (Kilinc and Karaer, 1995). Previous studies indicate that methanol extracts from aerial parts from sea knotgrass display *in vitro* antioxidant

* Corresponding author.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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and antimicrobial activities (El-Haci et al., 2013), and that it is endowed with different bioactive molecules, as for example quercetin, quercitrin and catechin (Kazantzoglou et al., 2009).

Ageing is a complex phenomenon characterized by a progressive accumulation of molecular damage in all constituent macromolecules, as for example nucleic acids, proteins and lipids, which leads to increased susceptibility to deterioration and death. The ineffectiveness and failure of maintenance and repair processes is the universal biochemical basis of ageing and age-related diseases including neurodegeneration. Alzheimer's (AD) is the most prevalent chronic neurological diseases and is expected to affect more than 60 million people in 2030 (WHO, 2014). AD is linked with a reduction of the levels of the neurotransmitter acetylcholine (ACh) as a result of the decreased activity of enzymes involved in its synthesis or excessive degradation by cholinesterases (ChE), such as acetyl- (AChE) and by butyrylcholinesterases (BuChE) (Duthey, 2013; Kumar et al., 2015). The inhibition of those enzymes results in an enrichment of the ACh levels in the synaptic cleft, leading to the improvement of cognitive function and consequent reduction in the disease progression (Kumar et al., 2015).

According to the free radical theory of aging the accumulation of oxidative damage over time is responsible for cellular aging and adds to the occurrence of age-related diseases (Harman, 1956; Aschbacher et al., 2013). The brain has a high lipid content and oxygen demand, which coupled with decreased antioxidative mechanisms makes it a constant source of reactive oxygen species (ROS) that contributes to neuronal damage (Uttara et al., 2009; Morales et al., 2014; Pasqualetti et al., 2015). Therefore, substances able to mitigate oxidative stress are acknowledged to have the potential to reduce the ageing-related physical and cognitive decay (Aschbacher et al., 2013). In addition, with ageing the immune system endures a process of senescence (immunosenescence), convoyed by a low-grade chronic proinflammatory environment in several tissues. This proinflammatory environment is designated as "inflammaging" (Franceschi et al., 2000, 2007), and is characterized by the accumulation of activated astrocytes and microglial cells that produce pro-inflammatory mediators, such as cytokines and ROS (Morales et al., 2014; Pasqualetti et al., 2015; Deleidi et al., 2015). Thus, discovery of novel molecules and/or products targeting age-linked inflammation may promote healthy brain aging and add for the prevention and/or amelioration of neurodegenerative and neuropsychiatric disorders (Deleidi et al., 2015).

There are several reports on the *in vitro* neuroprotective activity of different *Polygonum* species (Kim et al., 2010, 2013; Ahmad et al., 2014; Ayaz et al., 2015). For instance, root extracts from a related Polygonaceae species – *P. multiflorum* (He Shou Wu or Fo Ti, synonym for *Reynoutria* (*Fallopia*) *multiflora*) have been used in Traditional Chinese Medicine for its claimed anti-ageing benefits, and nowadays it is also marketed as a food supplement for its antioxidant and neuroprotective effects (Chan et al., 2003). However, there is no information regarding the potential of *P. maritimum* as a source of molecules with industrial applications as food additives and/or nutraceuticals with cognitive improvement properties. IIaving this in mind, this work evaluated for the first time the *in vitro* neuroprotective activity of dichloromethane and methanol extracts of aerial organs and roots of *P. maritimum*. For that purpose, samples were evaluated for their inhibitory activity against AChE and BuChE, ability to attenuate hydrogen peroxide (H_2O_2)-induced injury in human neuroblastoma SH-SY5Y cells, and also to reduce nitric oxide (NO) production in lipopolysaccharide (LPS)-induced neuroinflammation on N9 microglia cells. Additionally, the chemical profile of the extracts was established by ultra-high-pressure liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS). Finally molecular docking and molecular dynamics (MD) studies were performed on the identified compounds.

2. Materials and methods

2.1. Chemicals, culture media and supplements

Sodium nitrite, LPS from *Escherichia coli,* sulphanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride (NED), AChE (EC 3.1.1.7), BuChE (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine iodide, 5-thio-2-nitrobenzoate (DTNB) and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied phosphoric acid. Lonza (Belgium) provided Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RMPI) 1640 medium, fetal bovine serum (FBS), trypsin, L-glutamine and penicillin/streptomycin. Additional reagents and solvents were obtained from VWR International (Belgium).

2.2. Plant material

Samples from *P. maritimum* were collected in Ludo (South of Portugal) in June of 2013 (coordinates: 37° 0′ 50.681″ N 7° 59′ 3.516″ W), and the taxonomical classification was determined by Dr. Manuel J. Pinto (botanist from the National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). A reference specimen was kept in Marbiotech laboratory's herbarium (voucher n°. MBH22). Plants were divided in roots and leaves, cleaned, oven dried for 3 days at 40 °C, powdered and stored at −20 °C.

2.3. Extraction

Dried samples were mixed with methanol and dichloromethane (1:40, w/v) and extracted overnight at room temperature (RT, aprox. 20 °C), under stirring. After filtration (Whatman n° 4), extracts were evaporated under reduced pressure and temperature (< 40 °C), and then dissolved at the concentration of 20 mg/mL in dimethylsulfoxide (DMSO), for the biological assays, or in the corresponding solvent for the chemical analysis, and stored at −20 °C until needed.

2.4. Chemical profiling of the extracts by UHPLC–MS/MS

The extracts were dissolved in 1.5 mL of acidified HPLC-grade water (0.2% formic acid), centrifuged (10 × 1000 rpm, 10 min) and subjected to solid phase extraction (SPE) clean up to remove excess chlorophyll and residual substances (sugars).

2.4.1. SPE sample clean up

For the conditioning of SPE cartridges (Oasis HLB 3 cc Vac Cartridge, 60 mg Sorbent per Cartridge, 30 µm Particle Size, Waters Corporation, USA) the cartridge bed was washed with 5 mL of methanol, and with 5 mL of 0.2% aqueous formic acid. Then, the sample was loaded and washed with 5 mL of HPLC-grade water (eluate discarded after preliminary checking by UPLC), followed by elution with 80% methanol. The samples (of intense yellow color without chlorophyll), were re-evaporated to dryness under nitrogen flow and redissolved in methanol (25%, v/v, in water). Then, samples were filtered through 0.22 µm syringe filters and analyzed using UPLC–MS.

2.4.2. UPLC–MS/MS conditions

A Waters ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA), consisting of a binary pump system, sample manager, column manager and PDA detector (also from Waters Corp.) were used. The analytes were separated on an HSS Waters C18 column (100 mm × 2.1 mm i.d., 1.7 µm), with VanGuard Pre-column (HSS 5 mm × 2.1 mm i.d., 1.8 µm) both Waters Corp., Milford, MA, USA, which was maintained at 40 °C. The following solvent system: mobile phase A (0.1% formic acid in Milli-Q water, v/v) and mobile phase B (0.1% formic acid in pure MS gradient grade acetonitrile, v/v) was

applied. The gradient program was as follows: 0–0.5 min, 1% B; 0.5–10.0 min, 1–50% B; 10.0–11.0 min, 50–99% B; 11.0–12.0 min, 99% B, followed by 3 min column re-equilibration with 1% B. Samples were kept at 8 °C in the sample manager. The flow rate was adjusted to 0.40 mL/min. The injection volume of the sample was 3.0 µL (full loop mode) and samples were analyzed in triplicate. Strong needle wash solution (95:5, methanol–water, v/v) and weak needle wash solution (5:95, acetonitrile–water, v/v) were used. The detection wavelength for chromatographic profile in addition to acquisition of total ion flow was set at 325 nm at a 5 point/s rate, at 3.6 nm resolution and 250 nm at a 5 point/s rate, at 3.6 nm resolution. The separation was completed in 12 min. Peaks were assigned on the basis of their UV spectra, mass to charge ratio (m/z) and ESI–MS/MS fragmentation patterns. Waters MassLynx software v.4.1 was used for acquisition and data processing.

The tandem MS analyses were carried out on a TQD mass spectrometer (Waters Corp.) equipped with a Z-spray electrospray interface. The following instrumental parameters were used for ESI–MS analysis of polyphenolic compounds (negative ionization mode): capillary voltage, 2.99 kV; cone voltage, 40 V; desolvation gas, N_2 800 L/h; cone gas, N_2 100 L/h; source temp. 140 °C, desolvation temp. 350 °C. Compounds were analyzed in the full scan mode (mass range of 100–1600 amu was scanned). Identification was based on available literature data and matching of fragment ions

2.5. Inhibition of enzymes related with neurological diseases

2.5.1. AChE and BuChE inhibitory activity

The inhibitory effect of the extracts on AChE and BuChE was assessed by the Ellman method (Ellman et al., 1961) according to Custódio et al. (2015). Samples were tested at concentrations ranging from 0.04 to 1 mg/mL, and the results were expressed as percentage of inhibition relative to a control containing DMSO, and as IC_{50} values (mg/mL). Galanthamine was used as standard at concentrations up to 1 mg/mL.

2.6. Cell-based assay

2.6.1. Cell culture and cytotoxicity evaluation

The SH-SY5Y (human neuroblastoma) cell line was kindly provided by Dr. Eduardo Soriano (Barcelona Science Park, Spain), while N9 (mouse microglia) cells were provided by Dr. João Malva (Faculty of Pharmacy and Centre for Neurosciences and Cell Biology, University of Coimbra, Portugal). SH-SY5Y cells were maintained in DMEM culture medium, whereas the N9 cell line was cultured in RPMI-1640, both supplemented with 10% heat-inactivated FBS, 1% L-glutamine (2 mM), and 1% penicillin (50 U/ml)/streptomycin (50 µg/mL). Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2. Exponentially growing cells were plated in 96-well tissue plates at a density of 1×10^4 cells/well and incubated for 24 h. The extracts were then applied at different concentrations (3 − 100 µg/mL) for 24 h. Control cells were treated with DMSO at the highest concentration used in test wells (0.5%), and cell viability was determined by the MTT colorimetric assay (Mosmann, 1983) as described elsewhere (Rodrigues et al., 2014). Results were expressed in terms of cell viability (%) relative to the control. Non-toxic concentrations of the extracts, that is, those allowing cellular viability equal or higher than 80% were selected and used to evaluate their protective effect against H_2O_2 insult in neuronal cells.

2.6.2. Protective effect on hydrogen peroxide-induced cytotoxicity in SH-SY5Y cells

For the protective study, SH-SY5Y cells were seeded at a density of 5×10^4 cells/well in 96-well microplates and left to adhere overnight. Then two assays were performed: in the co-application assay, cells were simultaneously incubated with H_2O_2 and non-toxic concentrations of the extracts for 24 h. In the pre-incubation assay cells were treated with the extracts for 24 h, the extracts were removed, and cells were treated with 100 µL of H_2O_2 (4 mM) for 30 min (Custódio et al., 2015). In both assays, cellular viability was determined by the MTT assay after the incubation period (Mosmann, 1983). The stock solution of H_2O_2 was prepared in phosphate-buffered saline (PBS; pH 7.4) and diluted in DMEM without FBS immediately before use. Results were expressed in terms of cell viability (%) relative to control cells containing DMSO (0.5%, *v/v*) and H_2O_2.

2.6.3. Protective effect on LPS-induced neuroinflammation in N9 microglia cells

N9 cells were plated at a density of 2.5×10^5 cells/well in 96-well culture plates, and left to adhere overnight. Then, non-toxic concentrations of the extracts and the LPS (10 µg/mL) were diluted in serum- and phenol-free culture medium, applied to the cells and incubated for 24 h. The NO content was measured by the Griess assay, using a calibration curve made with sodium nitrite (1.5–100 µM) (Rodrigues et al., 2017). Results were expressed as a percentage of inhibition relative to a control containing DMSO (0.5%, *v/v*), and as IC_{50} values (µg/mL).

2.7. Molecular modeling

2.7.1. Ligand setup

The 2D structures of the compounds, identified in methanol and dichloromethane extracts of *P. maritimum* leaves and roots, (Fig. 1) were retrieved from ZINC ligand data base (Irwin and Shoichet, 2005) and then energy minimization was performed by OPLS2005 force field. The protonation states of the ligands were assigned at a physiological pH (pH 7.4) using Epik module of Maestro molecular modeling package (Greenwood et al., 2010). The electrostatic potential (ESP) charges of the atoms were calculated by Austin Model 1 (AM1) (Dewar et al., 1985). In order to search the conformers that have low energies of the studied compounds, MacroModel module of Maestro molecular modeling package was utilized and the selected ligands were then implemented into the simulations.

2.7.2. Molecular docking

All molecular docking simulations were performed using Induced Fit Docking (IFD) (Farid et al., 2006) protocol. This method was programmed to enhance the flexibilities of the amino acid at the binding sites. IFD approach comprises the following steps: i) the molecule was docked into the catalytic domain of the target using Glide/Standard Precision (SP), while the protein atoms were rigid (Friesner et al., 2006). Only complexes with high docking scores were forwarded to the next steps, ii) Amino acids of the complexes that were within 5 Å of the docked ligands were then refined; iii) Finally, the molecules were re-docked into the refined target using Glide/Extra Precision (XP) algorithm.

2.7.3. Molecular dynamics (MD) simulations

The top-IFD poses of the selected compounds from the lowest binding energies were submitted to the MD simulations. The complexes were hydrated in the cubic box with explicit TIP3P water molecules that have 10 Å thickness from surfaces of protein. The MD simulations were performed for apo and holo systems of both enzymes by Desmond code (Bowers et al., 2006). The particle-mesh Ewald method (Essmann et al., 1995) was used to calculate the long-range electrostatic interactions. A cut-off radius of 10.0 Å was used for both van der Waals and Coulombic interactions. Nose – Hoover thermostat (Evans and Holian, 1985) and Martyna-Tobias – Klein protocols (Martyna et al., 1996) were used to adjust the temperature and pressure of the systems at 310 K and 1.01325 bar, respectively. The time-step was assigned as 2.0 fs. The default values were used for minimization and equilibration steps and finally 100-ns production run were performed for each simulations. The details of the simulation protocols were described in the

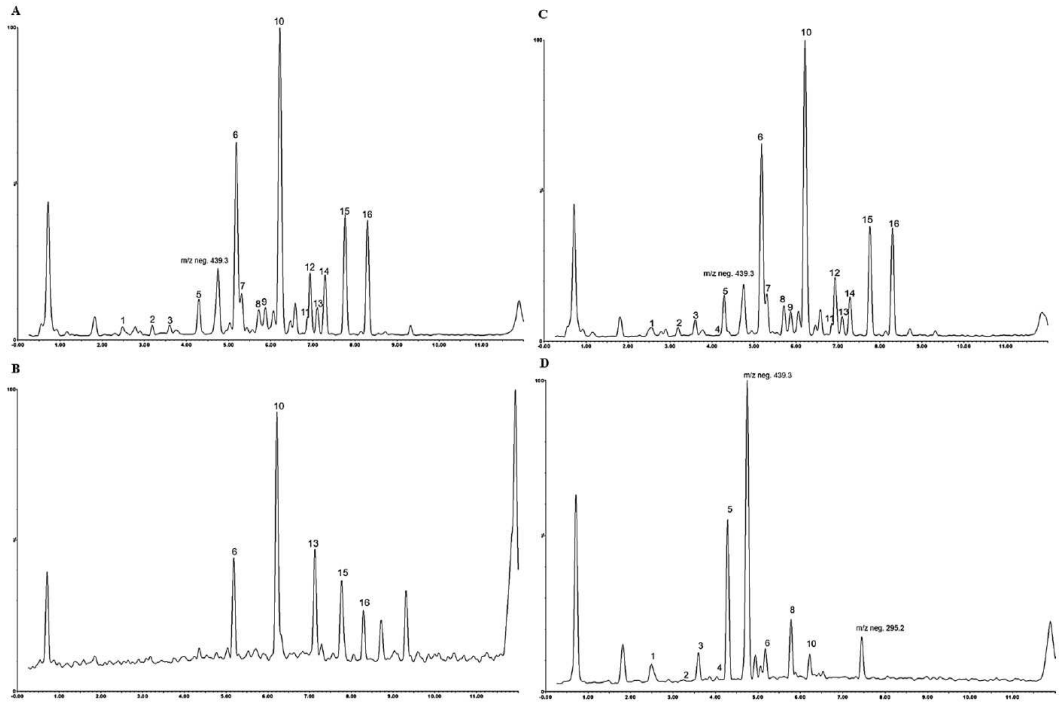

Fig. 1. UPLC–MS/MS analysis of methanol and dichloromethane extracts from leaves (A and B, respectively) and roots (C and D, respectively) of *P. maritimum*. Peak numbers refer to the compounds listed in Table 1.

previous studies (Durdagi et al., 2016; Salmas et al., 2017).

2.8. Statistical analysis

Results were expressed as mean ± 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 the Duncan's test. *P* values lower than 0.05 were considered significant. All statistical analyses were performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft corporation). The GraphPad Prism (v. 5.0) software was used to calculate the IC_{50} values through sigmoidal fitting of the data.

3. Results and discussion

3.1. Phytochemical characterization of the extracts

The chemical composition of *P. maritimum* extracts was assessed by comparison of the retention times and mass fragmentation pattern obtained by ULPC-MS/MS analysis, and results are summarized on Table 1. Fifteen phenolic compounds and one acetophenone [polygonophenone (**5**)] were identified. Among the phenolics, six were flavonol glycosides [quercetin-3-*O*-glucoside (**7**), avicularin (**8**), quercitrin (**9**), quercetin-3-*O*-glucuronide (**11**), myricetin 2"-*O*-acetyl-3-*O*-glucuronide (**12**) and europetin-3-*O*-rhamnoside (**14**)], three were flavan-3-ols [epigallocatechin (**1**), catechin/epicatechin (**3**) and epigallocatechin gallate (**5**)], one is a flavone glycoside [myricitrin (**6**)], and one is a proanthocyanidin [**2**]. Moreover, four compounds could not be precisely identified, namely one myricetin (**10**), two quercetin (**13**,**15**) and one isorhamnetin (**16**) derivatives. From these, 15 were detected in the methanolic extracts from leaves, (**1**–**4** and **6**–**16**) and roots (**1** and **3**–**16**), of which compounds **3**, **6** and **10** were the major components (Fig. 1 and Table 1). Less compounds were identified in the dichloromethane extracts, namely 3 in the leaves (**6**, **10**, **15**) and 5 in the roots (**1**–**5**) (Fig. 1 and Table 1). From the compounds detected in this study, catechin (**2**), polygonophenone (**5**), and quercitrin (**9**) had been previously identified in *P. maritimum* (Kazantzoglou et al., 2009). Although the flavonoid glycosides, such as avicularin (**8**) and quercitrin (**9**) have not yet been identified in this species, they have frequently been found in other *Polygonum* species, namely *P. hydropiper*, *P. aviculare*, *P. persicaria*, *P. lapathifolium*, *P. amphibium* and *P. convolvulus* (Smolarz, 2002).

Almost all the compounds identified in *P. maritimum* extracts were phenolics, which are widespread plant secondary metabolites known to participate in important physiological roles in plants, such as resistance to pathogens and herbivores, allelopathy, oxidative stress and plant growth regulation (Balasundram et al., 2006). Additionally, they have multiple beneficial properties for human health, namely as neuroprotective agents that may modulate cellular responses against oxidative stress and neuroinflammation (Kim, 2010). Epidemiologic studies have associated the moderate consumption of phenolic-rich foods and beverages, as for example tea, chocolate, red wine and red berries, with increased cognitive performance in the elderly population (Nurk et al., 2009; Willis et al., 2009). For instance, a long-term consumption of polyphenolic-rich berries had shown to improve the cognitive functions through, for example, the upregulation of the cholinergic system (Willis et al., 2009). Moreover, recent studies with rodents have shown that polyphenols, particularly flavonoids, can cross the blood brain barrier (BBB) and actually reduce neurodegeneration and improve the cognitive performance (Willis et al., 2009). In this sense, our results suggest that *P. maritimum* contains flavonoids with industrial uses as food additives and/or nutraceuticals for the amelioration of cognitive functions (Kim, 2010).

3.2. AChE and BuChE inhibitory activities

ACh is a crucial neurotransmitter for essential mental processes including memory and learning. Its concentration and functions decreases during ageing and at a drastic rate during the progression of neurological ailments, as for example AD (Francis, 2005; Gary and Wenk, 2006). Since ACh is hydrolyzed by the enzymes AChE and BuChE, their inhibition is considered a valuable tool to increase ACh

Table 1
List of compounds identified in methanol and dichloromethane extracts of *P. maritimum* leaves and roots, determined by UPLC–MS/MS analysis.

Peak no.[a]	RT (min)	Compound	[M-H]$^-$	Fragment	Sample			
					Methanol		Dichloromethane	
					Leaves	Roots	Leaves	Roots
1	2.51	Epigallocatechin	305	122,138,173,197	X	X		X
2	3.19	Procyanidin	577	289, 245	X			X
3	3.60	Catechin/Epicatechin	289	109,144,139,165,120,150	X	X		X
4	4.22	Epigallocatechin gallate	457	169,178,124		X		X
5	4.29	Polygonophenone	239	113,159,105	X	X		X
6	5.17	Myricitrin	463	316 271 287	X	X	X	X
7	5.32	Quercetin-3-*O*-glucoside	463	301,271,255,151,243	X	X		
8	5.75	Avicularin	433	301,271,255,283	X	X		
9	5.86	Quercitrin	447	301,271,163,239,151	X	X		
10	6.18	Myricetin derivative*	505	316,271,287,241,163	X	X	X	X
11	6.89	Quercetin-3-*O*-glucuronide	475	301,271,243,255	X	X		
12	6.94	Myricetin 2"-*O*-acetyl-3-*O*-glucuronide	533	316,286,271,163	X	X		
13	7.11	Quercetin derivative*	489	301,150,121	X	X	X	
14	7.29	Europetin 3-O-rhamnoside	477	315,283,331	X	X		
15	7.77	Quercetin derivative*	517	301,271,255,163	X	X	X	
16	8.29	Isorhamnetin derivative*	519	330,315,287,271	X	X	X	

[a] Corresponding peak number in the chromatogram on Fig. 1. X: presence of the compound.

Table 2
Acetyl- (AChE) and butyrylcholinesterase (BChE) inhibitory activities of methanol (MeOH) and dichloromethane (DCM) extracts of leaves and roots from *P. maritimum*. Results are expressed as IC_{50} values (mg/mL).

Species	Plant Part	AChE		BuChE	
		MeOH	DCM	MeOH	DCM
P. maritimum	Leaves	0.27 ± 0.01^c	0.91 ± 0.02^d	0.62 ± 0.03^b	> 1
	Roots	0.17 ± 0.01^b	> 1	0.61 ± 0.03^b	> 1
Galanthamine*		0.01 ± 0.00^a		0.32 ± 0.01^a	

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 different letters are significantly different according to the Duncan's test ($P < 0.05$).

* Positive controls; −: not tested; na: not active (< 10% of activity at 1 mg/mL).

levels and to improve cognitive functions (Greig et al., 2005). Having this in mind the sea knotgrass extracts were tested for their inhibitory capacity on AChE and BuChE and results are depicted on Table 2. The methanol extracts had a high inhibitory activity towards AChE, particularly the roots, which allowed for the lowest IC_{50} value (0.17 mg/mL). The methanol extracts also displayed the highest BuChE inhibition with IC_{50} values of 0.62 mg/mL and 0.61 mg/mL for leaves and root extracts, respectively. A high capacity to inhibit cholinesterases has been previously reported for other *Polygonum* species, as for example methanol leaf extracts of *P. minus* (Ahmad et al., 2014) and from leaves and roots of *P. sachalinensis* (Fan et al., 2011). Similar to the observed in this work essential oils from leaves of *P. hydropiper* also exhibited dual cholinesterase inhibition (Ayaz et al., 2015). The high cholinesterase inhibitory activity of these extracts can be related to the presence of different quercetin derivatives, such as quercetin-3-*O*-glucoside (**7**), avicularin (**8**), quercitrin (**9**), quercetin-3-*O*-glucoronide (**11**), and other two non-identified derivatives (**13**, **15**), since they have been previously described with strong cholinesterase inhibitory capacities (Jung and Park, 2007; Khan et al., 2009). Moreover, different acethophenone derivatives also had AChE and BuChE inhibition, which may indicate a potential cholinesterase inhibition of polygonophenone, one of the main compounds of the active extracts (Shen et al., 2013). Our results strongly suggest knotgrass as a source of bioactive molecules with dual cholinesterase inhibition, which can be useful to improve cognitive functions.

3.3. Protective effect on H_2O_2-induced SH-SY5Y neuroblastoma cells cytotoxicity

Oxidative damage is not the single cause of biological ageing, but it is considered pivotal for the aging process (Muller et al., 2007). It is accepted thus that controlling oxidative damage may result in a reduction of the physical and cognitive decay linked with aging (Aschbacher et al., 2013). In this context, and based on the promising results obtained with the inhibition of AChE and BuChE, the sea knotgrass extracts were further explored for their neuroprotective potential through cell based assays. For that purpose, samples were tested for *in vitro* protective effects against H_2O_2 induced-cytotoxicity on human neuroblastoma SH-SY5Y cells. This cell line is extensively used as a model for *in vitro* neuroprotection assays (Park et al., 2015), while H_2O_2 is used in this context as an inducer of oxidative stress since it is the main ROS produced by the brain in neurodegenerative processes (Pan et al., 2009).

In the co-application assay cells were simultaneously incubated with H_2O_2 and non-toxic concentrations of the extracts, that is, those allowing for more than 80% of cell viability. In the pre-incubation assay cells were first pre-incubated with the non-toxic samples and then challenged with H_2O_2. The co-application of sea knotgrass leaf and roots methanol extracts and H_2O_2 on SH-SY5Y cells resulted in a significant increase on cellular viability when compared to the cells only treated with H_2O_2 at all the concentrations tested (Fig. 2A), thus suggesting a protective effect of the extracts against the H_2O_2-induced cytotoxicity. This was not observed after application of the dichloromethane extracts (Fig. 2B). However, when cells were pre-treated with the extracts, the methanol extracts did not protected SH-SY5Y cells

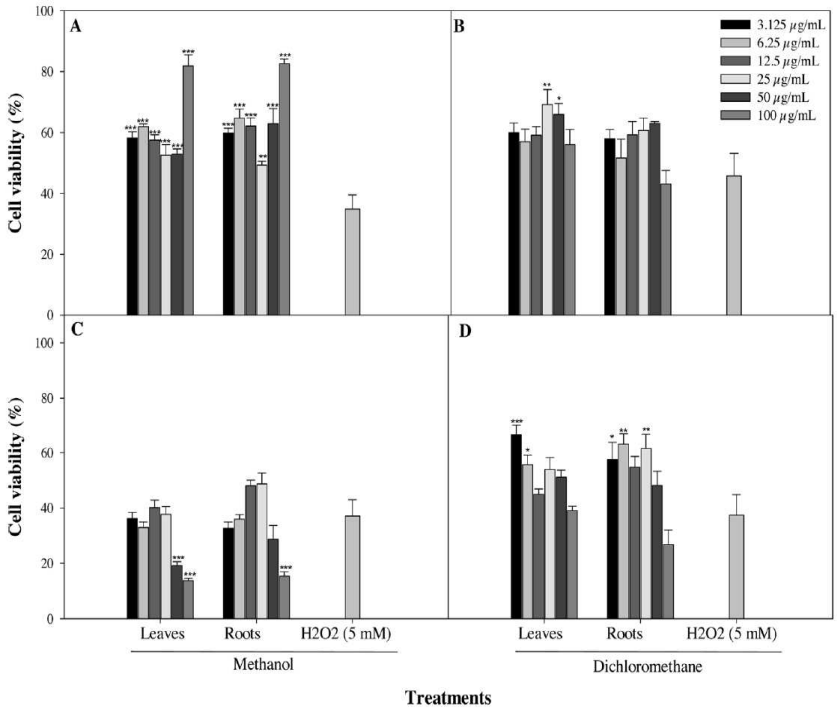

Fig. 2. Protective effect of methanol and dichloromethane extracts of leaves and roots from *P. maritimum* in SH-SY5Y neuroblastoma cells H_2O_2-induced cytotoxicity. Cells were incubated simultaneously with the extracts (methanol – A; dichloromethane – B) and 5 mM of H_2O_2 for 30 min, or pre-incubated with the extracts (methanol – C; dichloromethane – D) for 24 h before the application of 5 mM of H_2O_2 for 30 min. Values represent the mean ± standard error of the mean (SEM) of at least three experiments performed in triplicate (n = 9). Significantly differences from the H_2O_2 treated cells: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

from H_2O_2 cytotoxicity, whereas the dichloromethane increased the cellular viability at the lowest concentrations tested (3.125 and 6.25 μg/mL, Fig. 1C and D).

In a previous work, methanol extracts of *P. maritimum* had a strong *in vitro* capacity to scavenge different free radicals (Rodrigues et al., 2017). This antiradical capacity may explain the protective effect observed when the extracts and H_2O_2 were simultaneously applied to SH-SY5Y cells. Moreover, most of the compounds identified in the sea knotgrass extracts, in particular those of methanol, were phenolics. Thus, it is likely that these compounds are involved in the antioxidant activity, especially catechins (**1**–**5**) (Foti, 2007; Dai and Mumper, 2010) that were the major compounds identified in these extracts. Nevertheless, no apparent protective effect was obtained in the pre-treatment assay, suggesting that the antioxidant activity is occurring by direct reaction with the oxidant, and not through the activation of cellular antioxidant mechanisms. Besides, the pre-treatment with the dichloromethane extracts resulted in a higher protective activity than that obtained after application of the methanol ones, probably due to being constituted by less polar compounds that can easily enter the cells through their membranes than more polar compounds, with lower lipid solubility, which can activate the intracellular protective mechanisms (Chen, 2012). All together our results indicate that *P. maritimum*, especially the methanol extracts of leaves and roots, contain phytochemicals may counteract oxidative stress imposed by H_2O_2 treatment to neuronal cells *in vitro*.

3.4. Lipopolysaccharide (LPS)-induced neuroinflammation in N9 microglia cells

Microglia are the innate immune cells of the central nervous system (CNS) and are crucial for brain development and functioning (Kettenmann et al., 2011). These cells are activated during brain injury and neurodegenerative diseases releasing immune molecules such as cytokines, ROS, and growth factors. While activated microglia exert beneficial functions, as for example the phagocytic removal of pathogens and cellular debris, uncontrolled microglial activation that occurs with normal brain aging adds for neuronal dysfunction and death over time (Strohmeyer et al., 2002).

Microglia activation can be mimetized *in vitro* by the stimulation with the bacterial endotoxin LPS, which induces the expression of pro-inflammatory mediators, as for example IL-6 and NO (Liu et al., 2012). In this work, non-toxic extracts from *P. maritimum* were assessed for their *in vitro* anti-neuroinflammatory activity on LPS-stimulated N9 microglia cells. All samples caused a significant reduction on cellular viability at the concentration of 100 μg/mL (data not shown) and thus, this concentration was not used in the NO inhibitory assay. The application of 10 μg/mL of LPS on N9 cells induced the nitrite production from basal levels of 0.3 μM to around 3 μM (data not shown). This increase was significantly reduced in a dose-dependent manner by the application of all extracts of sea knotgrass particularly the methanol leaf extract that allowed for the lowest IC_{50} value (4.17 μg/mL; Table 3). Furthermore, all the tested extracts displayed anti-neuroinflammatory activity, with IC_{50} values significantly lower than that of the positive control (L-NAME; IC_{50} = 39.3 μg/mL) (Table 3).

Most of the compounds identified in the extracts have been reported previously with anti-inflammatory activity, namely epigallocatechin (**1**), catechin (**3**), myricitrin (**6**) and a quercetin derivative (**10**) that are the major compounds detected in the *P. maritimum* methanol extracts (Tipoe et al., 2007; Fan et al., 2011; Pereira et al., 2011). However,

Table 3
Inhibitory effect of methanol (MeOH) and dichloromethane (DCM) extracts of leaves and roots from *P. maritimum* on the NO production on LPS-stimulated N9 microglia cells. Results are expressed as IC_{50} values (μg/mL).

Species	Plant Part	Inhibition of NO production	
		MeOH	DCM
P. maritimum	Leaves	4.17 ± 0.20^{a}	14.2 ± 1.4^{bc}
	Roots	9.95 ± 2.77^{ab}	18.0 ± 1.5^{c}
L-NAME*		39.3 ± 3.9^{d}	

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 different letters are significantly different according to the Duncan's test ($P < 0.05$).

* Positive control.

Table 4
ADME analysis of the compounds.

Compound	QPlogKp	QplogPo/w	QPlogPw	QploghERG	QPlogKhsa	QPlogPC16	QPlogPoct	QPlogS	SASA	QPpolrz
Isorhamnetin	−4.56	1.12	12.46	−5.01	−0.2	10.3	17.61	−3.25	532.33	28.66
Europetin	−5.27	0.49	14.5	−4.84	−0.35	10.74	20	−2.92	537.93	28.36
Quercitrin	−5.8	−0.47	23.46	−5.17	−0.65	14.41	29.23	−2.99	658.39	38.26
Avicularin	−6.19	−0.87	23.4	−4.9	−0.73	14.22	27.85	−2.45	626.18	35.72
Isoquercitrin	−6.9	−1.48	26.72	**−5.34**	−0.89	15.47	31.81	−2.62	677.29	38.3
Myricitrin	−6.74	−1.16	25.71	**−5.31**	−0.79	15.06	32.4	−2.92	677.83	38.25
Epigallocatechin gallate	−7.44	−0.29	23.88	**−5.7**	−0.47	16.12	31.19	−3.47	690.9	39.28
Catechin	−4.67	0.41	15.55	−4.79	−0.44	10.42	19.86	−2.56	507.79	26.96
Procyanidin	−6.69	0.41	29.86	**−6.65**	−0.33	**19.88**	**39.42**	−4.54	815.34	51.37
Epigallocatechin	−5.48	−0.23	17.59	−4.67	−0.59	10.85	21.8	−2.33	517.62	26.72
Europetin 3-rhamnoside	−5.82	−0.35	23.82	**−5.35**	−0.67	14.92	30.05	−3.32	707.88	40.59
Quercetin 3-O-glucuronide	−7.46	−0.76	24.97	−3.49	−0.91	15.46	31.36	−2.88	675.7	39.07
Quercetin-3-glucoside	−6.73	−1.4	26.68	**−5.4**	−0.9	15.46	31.71	−2.67	681.66	38.48
Myricetin 2"-*O*-acetyl-3-*O*-glucuronide	**−8.96**	−1.17	28.94	−1.59	−1.28	17.35	34.5	−2.61	733.11	42.55

QplogKp: Predicted skin permeability, logKp (−8.0– −1.0); *QplogPw:* Predicted water/gas partition coefficient (4.0–45.0); *QplogPo/w:* Predicted octanol/water partition coefficient (−2.0–6.5); *QplogHERG:* Predicted IC_{50} value for blockage of HERG K^+ channels (below −5); *QplogKhsa:* Prediction of binding to human serum albumin (−1.5–1.5); *QplogPC16:* Predicted hexadecane/gas partition coefficient (4.0–18.0); *QplogPoct:* Predicted octanol/gas partition coefficient (8.0–35.0); *QplogS:* Predicted aqueous solubility, logS. S in mol dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (−6.5–0.5); *QPpolrz:* Predicted polarizability in cubic angstroms (13.0–70.0); *SASA:* Total solvent accessible surface area in square angstroms using a probe with a 1.4 Å radius (300.0–1000.0).

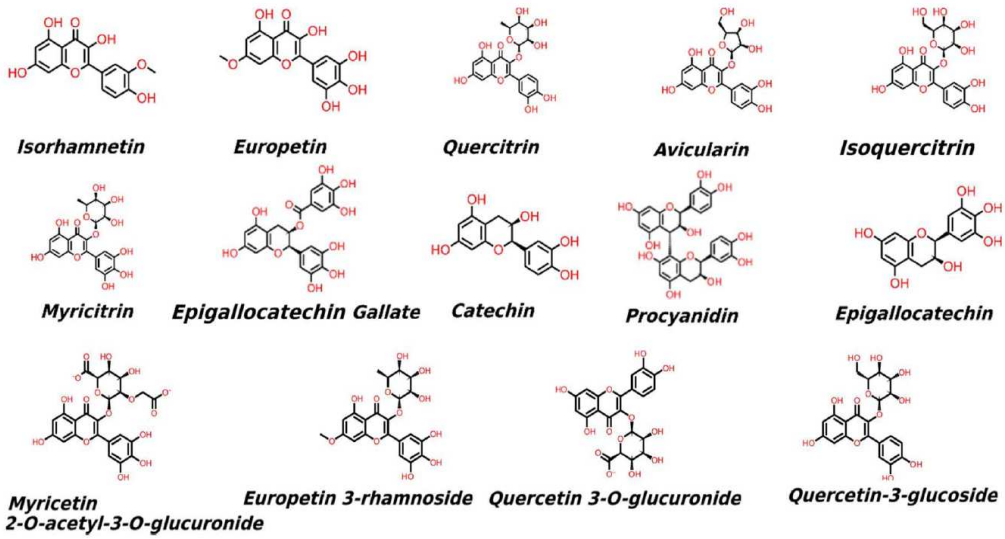

Fig. 3. 2D schematic views of the studied compounds.

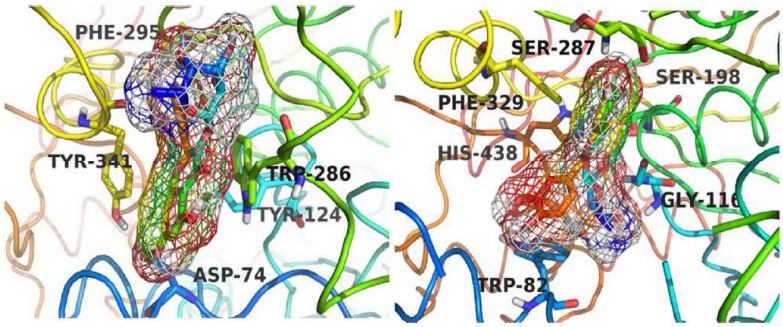

Fig. 4. The 3D schematic views of the quercitrin in complexes with the catalytic domains of the AChE and BChE, represented from MD trajectory frames-the key amino acids contributing to the main polar and non polar interactions with the ligand are monitored.

minor compounds such as a dimerous procyanidin (**2**), avicularin (**8**) and quercitrin (**9**) may also contribute to the observed activity, as well as other non-identified molecules (Vo et al., 2012; Bak et al., 2013). These compounds can be valuable as natural food additives and/or nutraceuticals with anti-inflammatory properties, and thus may contribute to reduce the progression of brain aging related disorders. Several *Polygonum* species have been traditionally used for the treatment of inflammation, and more than 15 species were already scientifically studied for their *in vitro* and *in vivo* anti-inflammatory properties, including *P. maritimum* (Soumyanath, 2005; Fan et al., 2011; Rodrigues et al., 2017). However, to the best of our knowledge, this is the first report describing the inhibitory ability of *Polygonum* species against neuroinflammation.

3.5. Absorption, distribution, metabolism and excretion (ADME)

ADME analyses of the identified compounds were evaluated using QikProp module of Maestro and the results are presented in Table 4. The main parameters in pharmacokinetic studies have been predicted, which comprise QplogKp, QplogPo/w, QplogPw, QplogS, QplogHERG, QplogKhsa, QplogPoct, QplogKhsa, QplogPC16 and SASA. The recommended values and list of abbreviations were given in the legend of Table 4. The data suggested that, except some of compounds (in bold; Table 4), all the results are within the stardard values of 95% of the approved drugs.

3.6. Binding energy assays

In order to determine the binding energies of the compounds against AChE and BuChE, IFD simulations were applied for the all studied molecules, in which the sufficient conformers with the lower energies were generated. The detailed views of results are presented (Figs. 3 and 4) for the individual complexes by color bar graphs, contrasting the energy changes, and furthermore the conformer populations of the complexes have been interpreted. These analysis are not aimed to compare the predicted binding energies between the compounds, but rather show their binding abilities to the targets. The favorable IFD scores of the most conformers suggested that all the compounds are capable of binding to AChE and BuChE proteins. Average IFD scores of procyanidin, which is observed as the highest potent compound are determined to be −9.54 kcal/mol and −10.38 kcal/mol in the catalytic domains of AChE and BuChE targets, respectively.

3.7. MD simulations

Since quercitrin and other similar derivatives were considered as main components of *P. maritimum* active extracts, which are described as potent AChE and BuChE inhibitors, these compounds were submitted to MD simulations in order to study their inhibition mechanisms in the catalytic domains and the conformational changes of the systems over 100 ns. Root mean square deviation (RMSD) of quercitrin atoms of their initial positions were calculated with two different aligning modes, based on the *Ca* and ligand heavy atoms. This strategy allow us to interpret the conformational changes and diffusion degrees of the ligands in both binding sites, as shown in Figs. 1S and 2S. The conformational stabilities of both compounds are evident in the profiles, in which the mean RMSD values are determined to be 2.14 Å and 0.82 Å for the AChE and BChE configurations, respectively (Fig. 3S). The difference among the values is mainly due to the high deviation from the docking pose in the period between 0 and 15 ns for the AChE case. Overall, the data suggested that the conformational and transition stabilities of the ligand are suitable to form strong interactions between the ligand and the active site amino acids. Furthermore, the pairwise RMSD calculations were applied to interpret the conformational change of the ligand in each frame relatively to all the other frames (Fig. 4S). The matrix profiles revealed the same results that after 15 ns the ligand has reached to sufficient equilibration. The 3D schematic views of the compounds in the binding pockets are represented from the MD trajectory frames, and the details of the poses are illustrated in Fig. 4, in which the key amino acids around the ligands contribute to the polar and non polar interactions are mentioned. Furthermore, the electron densities of the atoms are rendered to demonstrate the satisfactory complementarity between the ligands and targets. The data suggested that for the AChE complex, Asp74, Phe295, Tyr124, and Tryp286 are considered the main amino acids that stabilize the ligand through hydrogen and pi–pi stacking interactions. Trp82, Ser287, His438, Gly116, Phe329, Ser198, and Gly115 are the key residues for quercitrin in complex with BuChE. Not only the polar amino acids are essential to form the binding sites of these complexes, but also the hydrophobic resides showed a key role, which can be a shared feature between the two active sites.

3.8. Conclusions

This study describes for the first time the neuroprotective potential of methanol and dichloromethane extracts from leaves and roots of the halophyte *P. maritimum*. Compared to dichloromethane, methanol extracts had higher AChE and BuChE inhibitory activities, resulted in the highest increase on SH-SY5Y cellular viability after co-application with H_2O_2 and also the highest decrease of NO production on LPS-stimulated microglia. Furthermore, the UPLC–MS/MS analysis allowed for the identification of 15 bioactive polyphenols including flavonol glycosides flavan-3-ols, and proanthocyanidins. The molecular docking studies showed that all compounds may be able to bind to AChE and BuChE binding pockets. Altogether, these data indicate that the methanol extracts of *P. maritimum* may be a promising source of bioactive polyphenols able to be used as food additives and/or nutraceuticals as cognitive enhancers with anti-ageing potential.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.10.046.

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SUPPLEMENTARY MATERIAL

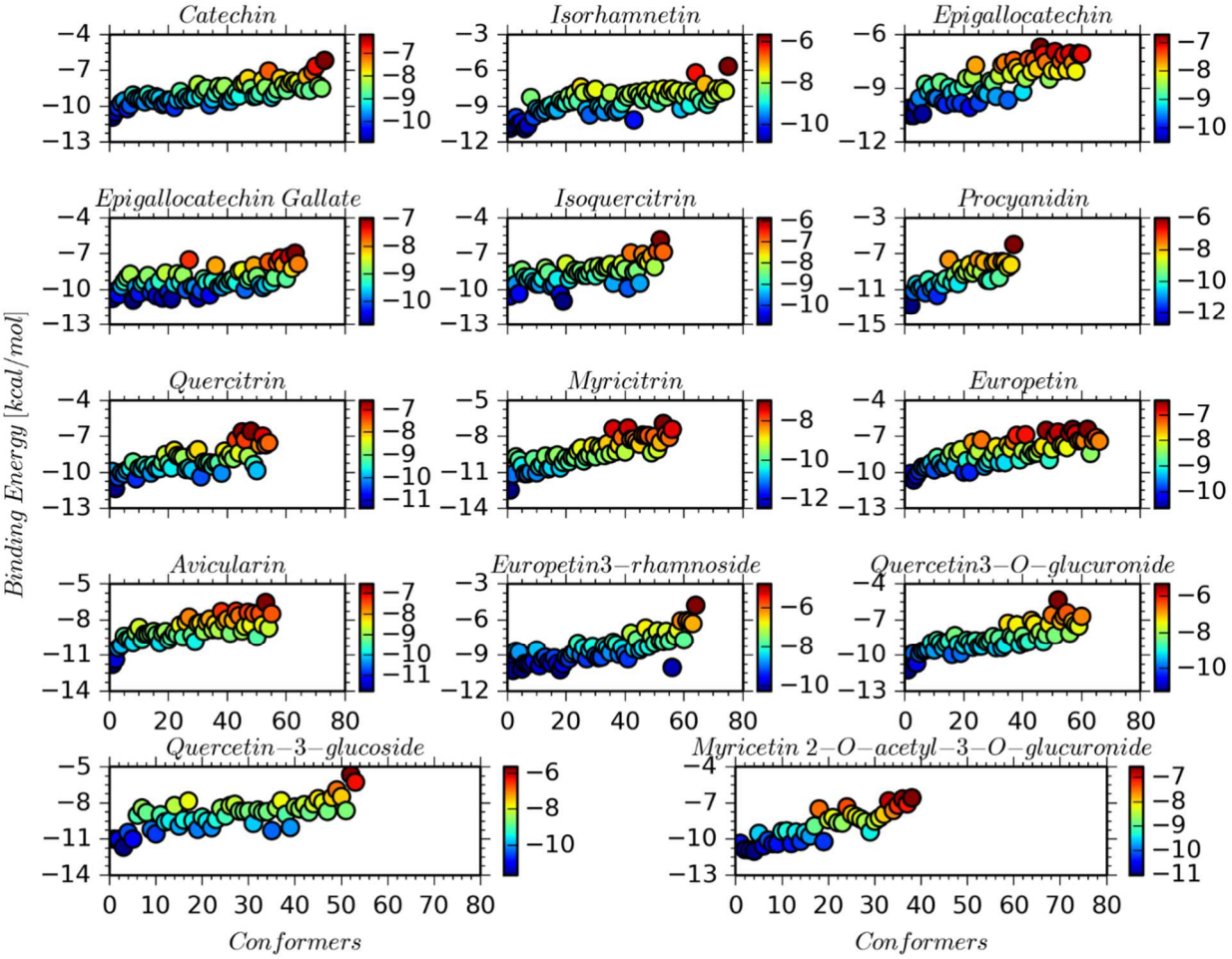

Fig. 1S. Binding energies of the compounds in the binding pockets of AChE, as obtained by IFD method.

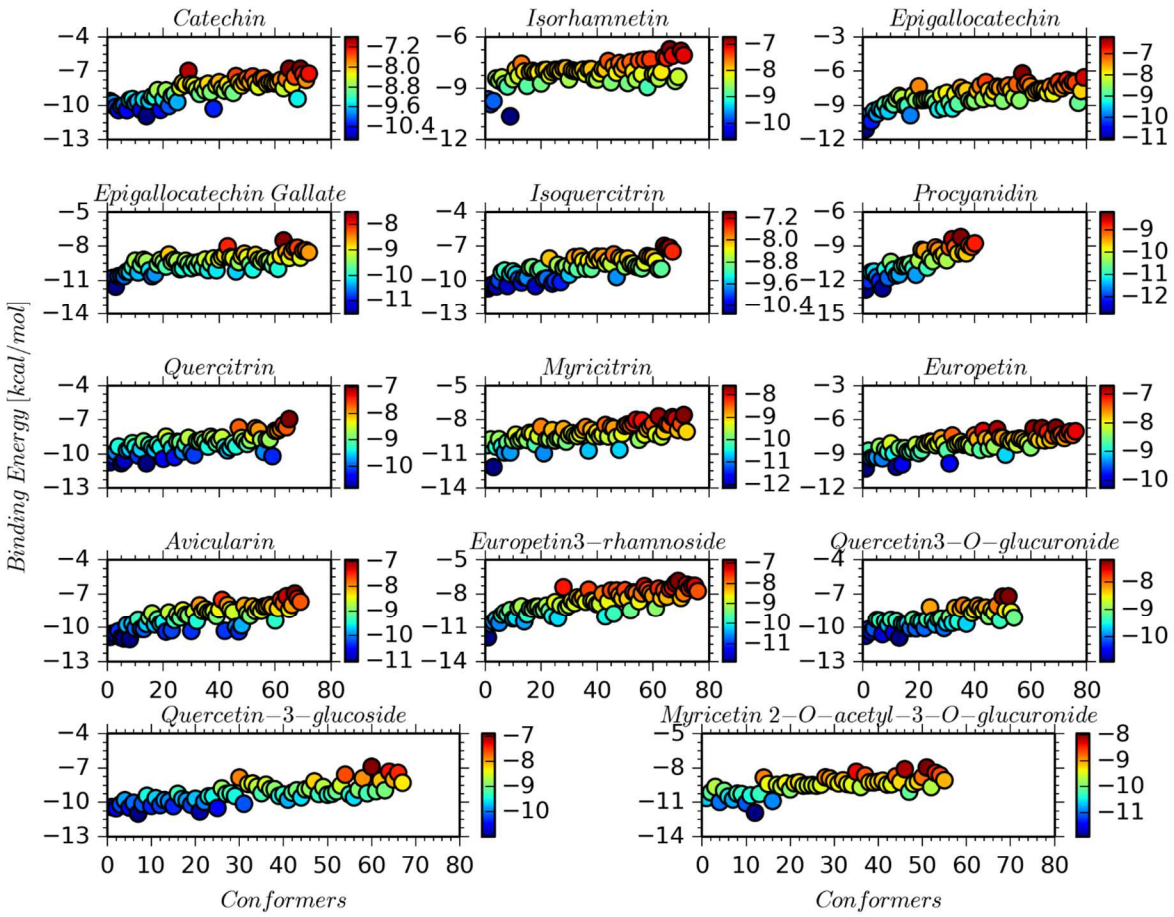

Fig. 2S. Binding energies of the compounds in the active sites of BuChE, as determined by IFD method.

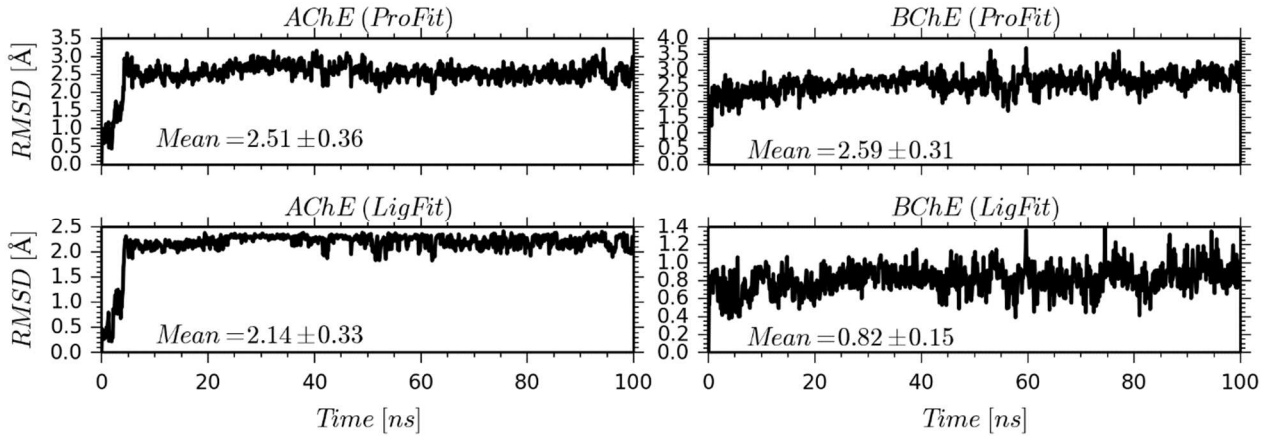

Fig. 3S. The RMSDs of the heavy atom quercitrin over 100 ns MD simulations respect to their starting positions. The calculations were performed with two modes, aligning of the systems based on the ligand (LigFit) and Ca atoms (ProFit).

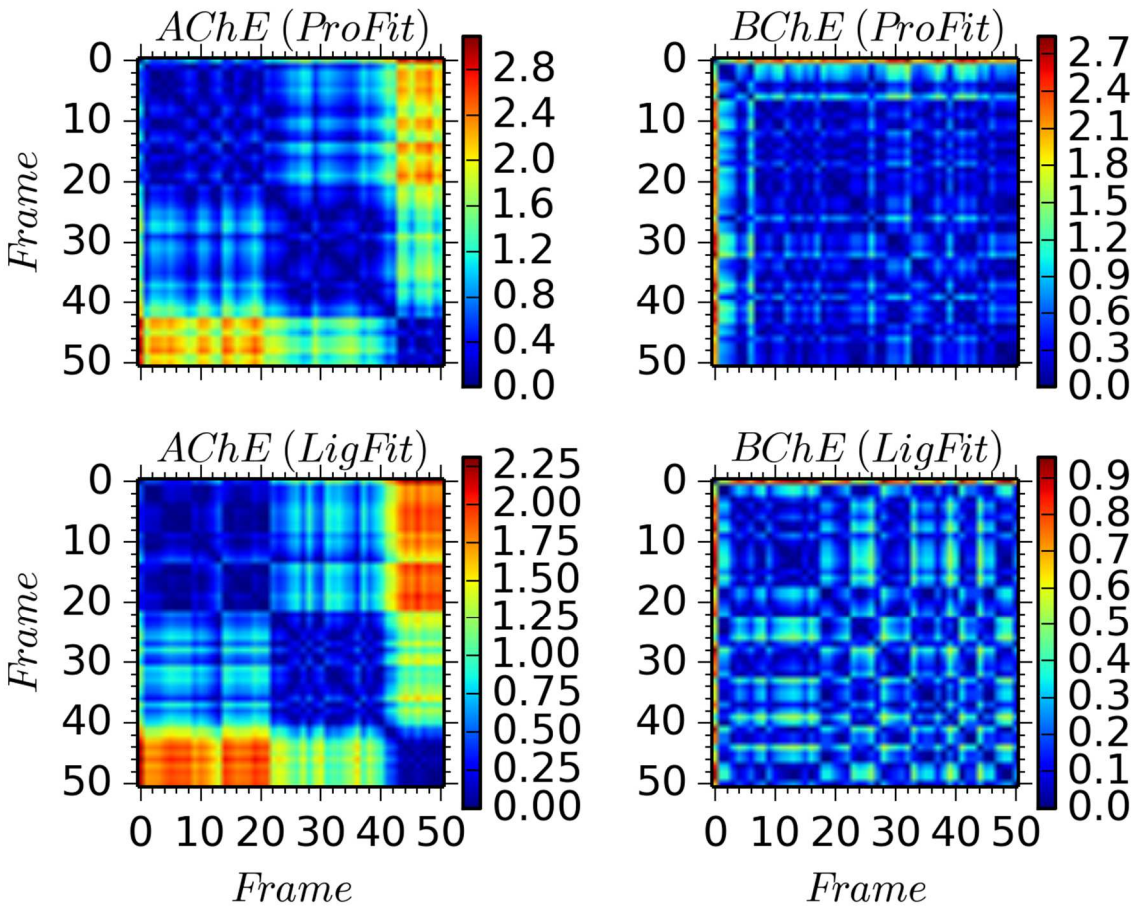

Fig. 4S. Pairwise RMSD calculations of the quercitrin atoms, in which each the RMSD of each MD frame respect to the other frames over 100 ns simulations have been interpreted.

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Sea knotgrass (*Polygonum maritimum* L.) as a potential source of innovative industrial products for skincare applications

Maria João Rodrigues[a], Adam Matkowski[b], Sylwester Ślusarczyk[b], Christian Magné[c], Thatyana Poleze[a], Catarina Pereira[a], Luísa Custódio[a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139, Faro, Portugal
[b] Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Wroclaw, Poland
[c] EA 2219 Géoarchitecture, UFR Sciences et Techniques, Université de Bretagne Occidentale, 6 Avenue V. Le Gorgeu, CS 93837, 29238 Brest Cedex 3, France

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ABSTRACT

Several *Polygonum* species and related genera display diverse skincare properties and were considered as active cosmetic ingredients. Thus, this work explored the halophyte sea knotgrass (*Polygonum maritimum* L.) as source of phenolics and flavonoids-enriched extracts with skincare properties, for the cosmetic industry. To improve the content in these compounds, different extraction solvents and methodologies were used, and the resulting extracts were tested for total contents of phenolics (TPC) and flavonoids (TFC). The acetone extract made on an ultrasound bath for 30 min had the highest TPC (275 mg of gallic acid equivalents [GAE]/g of dry weight [DW]), TFC (48.0 mg of rutin equivalents [RE]/g DW), and yield (20.3%). This extract was selected and evaluated for its *in vitro* antioxidant (total antioxidant, superoxide radical-scavenging and lipid peroxidation), anti-inflammatory (nitric oxide [NO] reduction on lipopolysaccharide [LPS]-stimulated RAW 264.7 macrophages), anti-wrinkles (elastase), anti-acne (lipase), antimicrobial (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*), anti-melanogenic (anti-tyrosinase and reduction of melanin production on B16 4A5 melanoma cells). The extract was also appraised for toxicity, and its chemical profile was determined by ultra-high-resolution mass spectrometry (UHRMS). The acetone extract showed a high $O_2^{-\cdot}$ scavenging (half maximal inhibitory concentration [IC_{50}] = 40.4 μg/mL), total antioxidant capacity (TAC; IC_{50} = 647 μg/mL), and late anti-lipid peroxidation (thiobarbituric acid [TBA]; IC_{50} = 784 μg/mL) properties. It also had a high capacity to reduce NO production on LPS-stimulated RAW 264.7 macrophages (IC_{50} = 22.0 μg/mL), and *in vitro* anti-melanogenic activity through tyrosinase inhibition (IC_{50} = 64.1 μg/mL), reduction of melanin production in B16 melanoma cells (IC_{50} = 77.7 μg/mL). It had low toxicity against mammalian cell lines. Thirty-one compounds were identified in the extract, including 24 flavonoids, 5 phenolic acids, and 1 acetophenone. Myricitrin, catechin and monogalloyl-hexose were the main compounds detected. Altogether, our results suggest sea knotgrass as a promising source of natural products with skincare properties for industrial cosmetic applications.

1. Introduction

The cosmetic industry is growing every year, and the global market value of cosmetics is estimated to achieve $429.8 billion in 2022 (Persistence Market Research, 2016; Business Wire, 2017). This is a highly innovative sector focused on science-driven novel products development, which allows to generate or redesign excellence and innovative ingredients corresponding to the ever-changing consumers' expectations. For instance, every year a quarter of all cosmetic products on the market are improved or are completely new. To correspond to this ever-increasing demand, research has been recurring to traditional natural ingredients to develop novel formulations, contributing to reducing the possible side-effects of the products and increasing their acceptance by the consumers (Cosmetics Europe, 2017). Following this trend, the natural cosmetics market is continuously growing and is expected to reach $22 billion by 2024 (Persistence Market Research, 2016; Business Wire, 2017). Among the global natural cosmetics segment, the share of natural skin care products is about 32.4%, followed by hair care (25%), make-up (22%) and oral products (13.7%) (Business Wire, 2017). Skincare products may have different applications, including for dryness, eczema, acne, free-radical scavenging, anti-inflammatory, anti-aging, skin lightening, skin protection and

* Corresponding author.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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sunscreens (Aburjai and Natsheh, 2003).

Usually, natural cosmetics incorporate ingredients from terrestrial plants, but nowadays marine organisms have started to be used as source of novel ingredients to develop increasingly innovative products, particularly those derived from micro- and macroalgae and marine bacteria (Guillerme et al., 2017). However, the marine coast also has a high diversity of vascular plants such as halophytes, which remain largely unexplored despite their high biotechnological potential (Guillerme et al., 2017). Marine halophytes are extremophile species that thrive in high salinity and ultraviolet (UV)-radiation environments, such as sand dunes or salt marshes, which contribute to extreme modifications in their physiology for their protection (Aslam et al., 2011). These conditions lead to the development of powerful morphological and physiological adaptations, as for example, antioxidant enzymatic defence systems, maintenance of ionic and osmotic homeostasis and synthesis of secondary metabolites, namely phenolic compounds that are one of the most important groups with human health benefits (Ksouri et al., 2012; Zengin et al., 2018). Beside their protective roles in plants, phenolics have a number of biological properties relevant for human health improvement, including antioxidant and anti-inflammatory, and due to their natural origin and low toxicity, are promising ingredients to be used in formulations with anti-ageing and skincare properties (Rodrigues et al., 2014, 2017, 2018a; Soto et al., 2015; Działo et al., 2016).

Various halophyte species exhibit bioactive properties relevant for skin care, such as antioxidant, anti-inflammatory and antimicrobial (*e.g. Pistacia lentiscus* L., *Crithmum maritimum* L., *Lythrum salicaria* L., *Limonium wrightii* (Hance) Kuntze., *Mesembryanthemum edule* L., *Limoniastrum monopetalum* (L.) Boiss., *Tamarix gallica* L. and *Salicornia ramosissima* J.Woods) which indicate the high potential of these plants for industrial cosmetic applications (Ksouri et al., 2012; Guillerme et al., 2017). The sea knotgrass (*Polygonum maritimum* L.) is a halophyte plant growing in the sandy coasts of Europe, Mediterranean and Black Sea regions and England (Kilinc and Karaer, 1995). Despite the previous reports indicating diverse biological activities of this species, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic and neuroprotective activities (El-Haci et al., 2013; Rodrigues et al., 2017, 2018a), there are no reports describing its potential cosmetic applications. However, extracts from other *Polygonum* species and related genera, such as *Fagopyrum esculentum* Moench. (syn. *P. fagopyrum* L.), *Persicaria bistorta* (L.) Samp. (syn. *P. bistorta* L.), Fallopia *japonica* (Houtt.) Ronse Decr. (syn. *P. cuspidatum* Sieb. & Zucc.) and *Fallopia multiflora* (Thunb.) Haralds. (syn. *P. multiflorum* Thun.), were considered active cosmetic ingredients with antioxidant, astringent, skin-conditioning, anti-inflammatory, anti-ageing, moisturizing and nourishing properties (NLM, 2012).

Several countries (EU, Brazil, India, Israel, New Zealand, Turkey, and UK) already abolished the *in vivo* testing in cosmetics, and many other are following this trend (Cosmetics Europe, 2017). Thus, cosmetic industry is limited to *in silico* and *in vitro* testing, which includes enzymatic and cellular models (Llorent-Martínez et al., 2016; Mocan et al., 2016; Cosmetics Europe, 2017). Having this in mind, in this work a sea knotgrass phenolic-enriched extract was evaluated for its potential as a cosmetic ingredient through the determination of its *in vitro* antioxidant (total antioxidant, superoxide radical-scavenging and lipid peroxidation), anti-inflammatory (NO reduction on LPS-stimulated RAW 264.7 macrophages), anti-wrinkles (elastase), anti-acne (lipase), antimicrobial (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*), and anti-melanogenic (tyrosinase and reduction of melanin production on B16 4A5 melanoma cells) activities. The extract was also evaluated for its toxicological properties against two mammalian cell lines (RAW 264.7 macrophages and B16 4A5 melanoma cells). The chemical profile of the extracts was established by ultra-high-resolution mass spectrometry (UHRMS).

2. Materials and methods

2.1. Chemicals, culture media and supplements

Lipase (EC 3.1.1.3), elastase (EC 3.4.21.36), tyrosinase (EC 1.14.18.1), 4-nitrophenyl dodecanoate (NPD), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA), N-[3-(2-Furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied Folin-Ciocalteau (F-C) phenol reagent, and all solvents used for chemical analyses. Additional reagents were obtained from VWR International (Belgium).

2.2. Plant material

The aerial parts (leaves and stems) from *P. maritimum* were collected in Fuseta island (South of Portugal) in August of 2017 (coordinates: 37° 2′ 33.079″ N 7° 44′ 47.321″ W). The botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) confirmed the taxonomical classification. A reference specimen was deposited in the Marbiotech laboratory herbarium (voucher nº. MBH22.1). The biomass was cleaned, freeze dried, powdered and stored at −20 °C.

2.3. Extraction

2.3.1. Selection of the best solvent for phenolic and flavonoid extraction

Dried biomass (500 mg) was mixed with 20 mL of different solvents (ethyl acetate, isopropanol, ethanol, acetone, water at room temperature, and water at 100 °C), and their mixtures with water (1:1, v/v), homogenized during 2 min (IKA Ultra-Turrax T10B), and extracted overnight at room temperature (RT, approx. 20 °C), under stirring. After filtration (Whatman nº 4), extracts were evaporated under reduced pressure and temperature (< 40 °C), and then dissolved at the concentration of 10 mg/mL in dimethyl sulfoxide (DMSO).

2.3.2. Selection of best extraction methodology for highest phenolic and flavonoid contents and yield

Dried biomass was extracted with acetone (1:40, w/v) using a Soxhlet apparatus during 5 h, and an ultrasonic bath for different periods (5, 15, 30 and 60 min). The extracts were filtered (Whatman nº 4), evaporated using a rotary evaporator, and dissolved at the concentration of 10 mg/mL in methanol.

2.4. Chemical composition

2.4.1. Determination of total phenolic (TPC) and flavonoid (TFC) contents

Samples (at the concentration of 10 mg/mL) were evaluated for TPC and TFC by F-C assay and aluminium chloride colorimetric methods, respectively, using a microplate reader (Biotek Synergy 4). Results were correspondingly expressed as gallic acid (GAE) and rutin (RE) equivalents in milligrams per gram of extract (dry weight, DW). All methods were performed as described in Rodrigues et al. (2015).

2.4.2. Plant material sample preparation

The acetone extract (100 mg) was dissolved in 1 mL of ultra-pure water (HLP10Uv, Hydrolab, Gdańsk) acidified with 0.2% formic acid and purified by Solid Phase Extraction (SPE) using C18 Sep-Pak cartridges (1 cm^3, 360 mg, Waters Corp., Milford, MA). The cartridges were washed with 0.5% methanol to remove carbohydrates, and then washed with 80% methanol to elute phenolics. The phenolic fraction was re-evaporated and dissolved in 1 mL 80% methanol (acidified with 0.2% formic acid). The sample (intense yellow colour without chlorophyll) was then centrifuged (23 000 x *g*, 5 min) and filtered (0.22 µm) before LC–MS analysis. All analyses were performed in triplicate for three independent samples (stored at −20 °C before analysis for no longer than 3 days).

2.4.3. Ultra-high-resolution mass spectrometry (UHRMS)

Liquid chromatography (LC) – electrospray ionization (ESI)-QTOF-MS estimation of polyphenol composition was carried out on a Thermo Dionex Ultimate 3000 RS (Thermo Fischer Scientific, Waltham, MA) chromatographic system, coupled to a Bruker Compact (Bruker, Billerica, MA) quadrupole time-of flight (QTOF) mass spectrometer, consisting of a binary pump system, sample manager, column manager and PDA detector. Separations were performed on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, USA), with mobile phase A consisting of 0.1% (v/v) formic acid in water and mobile phase B consisting of 0.1% (v/v) formic acid in acetonitrile. A linear gradient from 1% to 60% phase B in phase A over 20 min was used to separate phenolic compounds. The flow rate was 0.4 mL/min and the column was held at 30 °C. Spectra were acquired in negative-ion mode over a mass range from *m/z* 100 to 1500 with 5 Hz frequency. Operating parameters of the ESI ion source were as follows: capillary voltage 3 kV, dry gas flow 6 L/min, dry gas temperature 200 °C, nebulizer pressure 0.7 bar, collision radio frequency 700.0 V, transfer time 100.0 µs, and pre-pulse storage 7.0 µs. Ultrapure nitrogen was used as drying and nebulizer gas, and argon was used as collision gas. Collision energy was set automatically from 15 to 75 eV depending on the *m/z* of fragmented ion. Acquired data were calibrated internally with sodium formate introduced to the ion source at the beginning and ending of each separation *via* a 20 µL loop. Processing of spectra was performed with Bruker DataAnalysis 4.3 software.

2.5. In vitro skin care properties

2.5.1. Radical scavenging activity (RSA) on superoxide (O_2^-) radical

The superoxide RSA was tested on samples at concentrations ranging from 10 to 1000 µg/mL, by the method described in Rodrigues et al. (2016). Catechin was used as standard at the same concentrations of the samples, and results were expressed as percentage of inhibition, relative to a negative control containing methanol in place of the sample, and as half maximal inhibitory concentration (IC_{50} values, µg/mL).

2.5.2. Ferric thiocyanate (FTC) and thiobarbituric acid (TBA) tests

The inhibition of lipid peroxidation (FTC and TBA) was measured on samples at concentrations ranging from 10 to 1000 µg/mL, according to the method described by Bouaziz et al. (2015) with some modifications as previously reported (Rodrigues et al., 2017). The TBA test was conducted on the final day of FTC test as described before (Rodrigues et al., 2017). BHT was used as standard (10–1000 µg/mL), and results were expressed as percentage of inhibition, relative to a blank containing methanol, and as IC_{50} values (mg/mL), for those extracts exhibiting inhibitions higher than 50% when tested at 1000 µg/mL.

2.5.3. Total antioxidant capacity (TAC)

Total antioxidant activity was determined through the phosphomolybdenum assay using the method described by Prieto et al. (1999). Samples (100 µL; 10 to 1000 µg/mL) were mixed with 1 mL of phosphomolybdenum reagent (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulphuric acid), and incubated for 90 min in a water bath at 95 °C. After cooling to room temperature, the absorbance was read at 695 nm (Biotek synergy 4). Increased absorbance of the reaction mixture indicated increased TAC. Ascorbic acid was used as positive control at the concentration of 500 µg/mL, and results were expressed as percentage of inhibition relative to ascorbic acid at the previous concentration, and as IC_{50} values (µg/mL).

2.5.4. In vitro inhibition of tyrosinase (TYRO)

Extract and positive control (arbutin) were tested at concentrations between 10 and 1000 µg/mL, on the TYRO inhibitory activity as reported before (Rodrigues et al., 2018b). Results were expressed as a percentage of inhibition relative to a control containing methanol, and as IC_{50} values (µg/mL).

2.5.5. In vitro inhibition of elastase

Samples and standard (catechin) were tested at concentrations between 10 and 10,000 µg/mL on the elastase inhibitory activity as described in Rodrigues et al. (2018b). Results were expressed as a percentage of inhibition in relation to a control with methanol, and when possible as IC_{50} values (µg/mL), for extracts showing an inhibition higher than 50% at the highest concentration tested.

2.5.6. In vitro inhibition of lipase

Samples and positive control (orlistat) were tested, at concentrations ranging between 10 and 10,000 µg/mL, on the inhibitory activity of porcine lipase as previously described by Rodrigues et al. (2018b). Results were expressed as a percentage inhibition relative to a control containing methanol.

2.5.7. Antimicrobial activity

Antimicrobial activity was screened against three potent contaminants of cosmetic products, including the Gram-positive *Staphylococcus aureus* subsp. *aureus* (ATCC 33862), the Gram-negative *Escherichia coli* (ATCC 4157) and *Pseudomonas aeruginosa* (BCC), and the yeast *Candida albicans* (BCC). Bacterial strains were grown in liquid nutrient broth (Difco Surrey, England) at 37 °C (23 °C for the yeast) for 24 h before being used. A microplate-bioassay (microdilution) was used to study the antimicrobial activities of plant extract. Aliquots of each extract, corresponding to 1, 10 or 100 µg of dried extract, were dropped in sterile 96-well plates. After complete evaporation of the solvent, 100 µL of microorganism suspensions (10^2 cells/mL) obtained by dilution from the culture tube (10^8 cells/mL) were added to each well. Microbial suspension was used alone as positive control or in the presence of antibiotic mixture (5 mg/mL streptomycin and 10 mg/mL penicillin G) as negative control. Then, the microplate was aseptically sealed, agitated and incubated for 24 h at either 23 °C (yeast) or 37 °C (bacteria). Finally, microorganism growth was estimated by reading the absorbance in each well at 405 nm with a microplate spectrophotometer (Multiskan MCC/340, Titertek). Antimicrobial activity was expressed in percentage of growth inhibition using the following formula: Growth inhibition (%) = 100 − [100 * (Asample-A < C) / (AGC-A > C)], where A < C and A > C are the absorbances of the negative and of the positive control, respectively.

2.5.8. Cell culture and toxicity of the extract

The murine RAW 264.7 leukemic monocyte-macrophage cell line was obtained from Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal), and B16 A45 murine melanoma cell line were purchased from Sigma-Aldrich (Germany). RAW 264.7 macrophages and B16 4A5 melanoma cells were respectively maintained in RPMI 1640 and DMEM culture media supplemented with 10% heat-inactivated FBS, 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL) / streptomycin (50 µg/mL) and were kept at 37 °C in moistened atmosphere with 5% CO_2. Exponentially growing cells were plated in 96-well tissue plates at a density of 1×10^4 cells/well (RAW 264.7) and 2×10^3 cells/well (B16 4A5), followed by 24 h incubation. Samples were then applied at different concentrations (3 to 100 µg/mL) for 24 h or 72 h, for RAW 264.7 and B16 4A5, correspondingly. Control cells were treated with DMSO at the highest concentration used in test wells (0.5%), and cell viability was determined by the MTT colorimetric assay (Biotek Synergy 4), as described previously (Rodrigues et al., 2014). Results were expressed in terms of cellular viability (%).

2.5.9. In vitro anti-inflammatory activity on LPS-stimulated RAW 264.7 macrophages

Nitric oxide production was evaluated using RAW 264.7

macrophages as described by Rodrigues et al. (2014). Cells were plated at 2.5×10^5 cells/well in 96-well tissue plates, left to adhere overnight and treated with concentrations that permitted cellular viability above 80%, in serum- and phenol-free culture medium, with 100 ng/mL of LPS, for 24 h. Nitric oxide production was measured by the Griess assay, as reported before (Rodrigues et al., 2014). Results were expressed as percentage (%) of NO production, relative to a control containing DMSO (0.5%, v/v), and when possible as IC_{50} values (μg/mL).

2.5.10. In vitro inhibition of melanin production on B16 4A5 melanoma cells

The cellular melanin content was evaluated using B16 4A5 melanoma cells as described by Bouzaiene et al. (2016) with some modifications. Cells were plated at 3.5×10^4 into 12-well plates, allowed to adhere for 24 h, and then treated with concentrations that permitted cellular viability higher than 80% for 72 h. After treatment, adherent cells were trypsinized, and solubilized in 1 mL of sodium dodecyl sulphate (SDS; 1%, v/v). The absorbance of the samples was measured at 475 nm, and the melanin content was estimated using a standard curve of synthetic melanin (0–25 μg/mL).

2.6. Statistical analyses

Results were expressed as mean ± standard error of the mean (SEM), and tests were performed in triplicate. Significant differences were determined through analysis of variance (ANOVA) followed by the Tukey HSD test, or the Kruskal-Wallis test if parametricity was not found. When P values were less than or equal to 0.05, differences between samples were significant. All statistical analyses were performed using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft corporation). The IC_{50} values were determined by sigmoidal fitting of the data using the GraphPad Prism v. 5.0 program.

3. Results and discussion

3.1. Extraction optimization

Sea knotgrass is a rich source of phenolic compounds, particularly flavonoids (Rodrigues et al., 2017, 2018a) with multiple biotechnological applications, including as cosmetic ingredients. An improved phenolics extraction is usually performed using different polar solvents (*e.g.* ethanol, acetone and water), their combinations with water, and diverse extraction methods can also be used (Qasim et al., 2016). However, the phenolics extraction through these different solvents is also dependent of the species or biomass characteristics (Buhmann and Papenbrock, 2013; Qasim et al., 2016). In this work different extraction solvents (Table 1) and extraction methods (Table 2) were used to obtain sea knotgrass extracts enriched with phenolic and flavonoids. First, the biomass was extracted with ethyl acetate, isopropanol, ethanol, acetone, water at RT, and water at 100 °C, and their mixtures with 50% of water. The pure acetone extract had the best combination of total phenolics (240 mg GAE/g DW) and flavonoids (49.0 mg RE/g DW) contents (Table 1). In fact, acetone has already been described as an excellent solvent for phenolics and flavonoids extraction from plants (Munhoz et al., 2014; Tan et al., 2014). Thus, acetone was selected for testing different extraction methods, such as Soxhlet (5 h) and ultrasounds (5, 15, 30 and 60 min) to maximize the levels of phenolics and flavonoids and the extraction yield. Compared to the first extraction, ultrasonic extraction allowed an increase in the total phenolics (275 GAE/g DW), maintained the flavonoids content (48.0 mg RE/g DW) and doubled the extraction yield (20.3%; Table 2). Particularly, the flavonoid content had a time-dependent increase up to 30 min, whereas TPC had no significant modification. However, despite the highest yield, after the 60 min extraction both TPC and TFC decreased, probably due to the heating caused by ultrasounds that contribute to the degradation of some compounds (Ma et al., 2008). Ultrasounds increase the surface contact between solvents and samples and the permeability of cell walls, enabling the compounds release, and is a simple and low-cost technology (Azwanida, 2015). Other authors also showed that ultrasonic extraction can be an interesting choice to reduce extraction time coupled with higher yields, when compared to traditional maceration extraction procedures (Trusheva et al., 2007; Dhanani et al., 2017). Therefore, the acetone 30 min ultrasonic extract was selected to pursue the next experiments (chemical analysis and the *in vitro* skin care properties), since it allowed to have similar TPC and TFC, with highest yield and reduced extraction time.

Table 1
Total contents of phenolics (TPC) and flavonoids (TFC) of sea knotgrass (*P. maritimum*) aerial parts extracted with different solvents.

Extract	TPC (mg GAE/g DW)	TFC (mg RE/g DW)	Yield (%)
Ethyl acetate	37.3 ± 2.3^{e}	48.7 ± 0.9^{a}	5.00
50% ethyl acetate	133 ± 3^{c}	34.4 ± 1.2^{b}	13.4
Isopropanol	78.3 ± 5.0^{d}	11.7 ± 0.4^{e}	23.6
50% isopropanol	203 ± 2^{b}	21.8 ± 0.5^{cd}	30.8
Acetone	240 ± 4^{a}	49.0 ± 1.1^{a}	11.0
50% acetone	237 ± 4^{a}	24.3 ± 0.2^{c}	29.2
Ethanol	241 ± 4^{a}	32.5 ± 0.6^{b}	16.4
50% ethanol	227 ± 6^{a}	19.0 ± 0.2^{d}	20.4
Water RT	60.9 ± 3.4^{d}	10.2 ± 0.4^{e}	2.8
Water 100 °C	73.2 ± 3.6^{d}	11.6 ± 0.5^{e}	5.6

Values represent the mean ± 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). DW: dry weight; RT: room temperature; GAE: gallic acid equivalents; RE: rutin equivalents; TPC: mg GAE/g DW; TFC: mg RE/g DW.

Table 2
Total contents of phenolics (TPC) and flavonoids (TFC) of acetone extracts of sea knotgrass (*P. maritimum*) aerial parts using different extraction methods.

Extraction	TPC (mg GAE/g DW)	TFC (mg RE/g DW)	Yield (%)
Ultra sounds			
5 min	273 ± 4^{a}	38.5 ± 0.5^{d}	16.2
15 min	271 ± 2^{a}	45.9 ± 0.7^{b}	19.5
30 min	275 ± 3^{a}	48.0 ± 0.4^{a}	20.3
60 min	259 ± 3^{b}	42.1 ± 0.4^{c}	25.1
Soxhlet 5h	156 ± 7^{c}	43.9 ± 0.3^{c}	27.8

Values represent the mean ± 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). DW: dry weight; RT: room temperature; GAE: gallic acid equivalents; RE: rutin equivalents; TPC: mg GAE/g DW; TFC: mg RE/g DW.

3.2. Chemical composition

The chemical composition of the selected acetone extract from sea knotgrass was assessed by comparison of the retention times and mass fragmentation pattern obtained by ultra-high-resolution mass spectrometry (UHRMS) analysis, and results are summarized on Table 3 and Fig. 1. Thirty-one compounds were identified in the extract, including 24 flavonoids (**1**, **4–8**, **10–11**, **13**, **16–31**), 5 phenolic acids (**2**, **3**, **9**, **12** and **14**) and 1 acetophenone (**15**). Among these, several compounds were previously reported in this species, namely gallic acid (**3**), epigallocatechin (**6**), catechin (**11**), proanthocyanidin (**13**), polygonophenone (**15**), epicatechin (**16**), epigallocatechin gallate (**19**), myricitrin (**28**), and quercitrin (**31**) (Kazantzoglou et al., 2009; Rodrigues et al., 2017, 2018a, 2018b). Therefore, 22 compounds were identified in *P. maritimum* extracts for the first time here, including 4 phenolic acid derivatives (monogalloyl-hexose [**1**], mudanoside B [**2**], p-coumaric acid-hexose ester [**9**], one non-identified caffeic acid derivative [**12**], and 2-(β-D-glucopyranosyloxy)-3-phenylpropanoic acid [**14**]), 7 catechin derivatives (gallocatechin [**5**], (-)-epicatechin-O-

Table 3
List of compounds identified in the acetone extract of sea knotgrass (*P. maritimum*) aerial parts, determined by UHRMS analysis.

Peak no.[a]	Compound	RT (min)	m/z [M-H]-	Error [ppm]	Formula	MS^2 ion
1	Monogalloyl-hexose	0.70	331.0669	0.1	$C_{13}H_{16}O_{10}$	169,271,211,151
2	Mudanoside B	0.80	463.1093	0.1	$C_{18}H_{24}O_{14}$	301,169
3	Gallic acid	0.90	169.0136	0.1	$C_7H_7O_5$	125.00
4	Glucogallin	1.30	331.0676	1.1	$C_{13}H_{16}O_{10}$	169.00
5	Gallocatechin	2.20	305.0665	0.6	$C_{15}H_{14}O_7$	165,219,261,125
6	Epigallocatechin	6.10	305.0665	0.6	$C_{15}H_{14}O_7$	165,219,261,125
7	(-)-Epicatechin-O-Hexose	6.15	451.1226	−0.1	$C_{21}H_{24}O_{11}$	289,245,205,137
8	Dimeric procyanidin	6.20	577.1352	0.4	$C_{30}H_{26}O_{12}$	451,289,245,161,125
9	p-Coumaric acid-hexose ester	6.30	325.0925	1.4	$C_{15}H_{18}O_8$	163,119
10	3-O-Caffeoyl-D-glucopyranose	6.40	341.0877	0.5	$C_{15}H_{18}O_9$	179,135
11	Catechin	6.45	289.0725	0.3	$C_{15}H_{14}O_6$	245,203,151,179
12	Caffeic acid derivative	6.50	258.9921	−0.1	$C_{15}H_8O_{12}$	179,135
13	Proanthocyanidin	6.60	761.1367	−1.0	$C_{37}H_{30}O_{18}$	423,305,177,125
14	2-(β-D-Glucopyranosyloxy)-3-phenylpropanoic acid	6.70	327.1084	0.6	$C_{15}H_{20}O_8$	165,147
15	Polygonophenon	7.20	239.0591	3.6	$C_{12}H_{16}O_5$	159, 193,261[M + NA-H]
16	Epicatechin	7.27	289.0719	1.2	$C_{15}H_{14}O_6$	245,203,151,179,125
17	Epigallocatechin-(4β- > 8)-epicatechin 3-O-gallate	7.30	745.1426	−3.1	$C_{37}H_{30}O_{17}$	407,289,177
18	Dimeric galloylated proanthocyanidin - isomer of 17	7.32	745.1426	−2.5	$C_{37}H_{30}O_{17}$	423,305,269,161
19	Epigallocatechin gallate	7.50	457.0785	0.4	$C_{22}H_{18}O_{11}$	169,305,125
20	Myricetin 3-galactoside	7.52	479.0869	1.1	$C_{21}H_{20}O_{13}$	316.00
21	Myricetin 3-(-galloylgalactoside)	7.80	631.0964	1.2	$C_{28}H_{24}O_{17}$	479,316
22	Dimeric procyanidin	7.90	577.1355	−0.6	$C_{30}H_{26}O_{12}$	289,407,245,161
23	Dimeric procyanidin-O-gallate	7.95	729.1473	−1.7	$C_{37}H_{30}O_{16}$	407,289,169,271,245,161
24	Myricetin-O-hexoside	8.10	479.0843	−1.2	$C_{21}H_{20}O_{13}$	316.0218,317
25	Tetrahydroxyflavanonol-O-hexoside	8.30	465.1045	−0.6	$C_{21}H_{22}O_{12}$	313,303.0527,151
26	Quercetin-galloylgalactoside	8.40	615.1022	−0.6	$C_{28}H_{24}O_{16}$	463,300,241,169,519
27	Epicatechin gallate	8.50	441.0822	1.1	$C_{22}H18O_{10}$	289,169,245,137
28	Myricitrin	8.55	463.0909	−2.8	$C_{21}H_{20}O_{12}$	316.0238, 179
29	Isomer of 25	8.60	465.1045	−0.6	$C_{21}H_{22}O_{12}$	313.0930,303.0527,151
30	Phlorizin	8.67	597.1826	−0.2	$C_{27}H_{34}O_{15}$	357,387,463,417,316,273
31	Quercitrin	9.25	447.0945	−0.1	$C_{21}H_{20}O_{11}$	300,301,284,255

[a] Corresponding peak number in the chromatogram on Fig. 1.

hexose [**7**], 2 epigallocatechin-(4β- > 8)-epicatechin 3-O-gallate isomers [**17** and **18**], 3 dimeric procyanidins [**8**, **22** and **23**], and epicatechin gallate [**27**]), 3 myricetin derivatives (myricetin 3-galactoside [**20**], myricetin 3-(-galloylgalactoside) [**21**] and myricetin-O-hexoside [**24**]), 1 quercetin derivative (quercetin-galloylgalactoside [**26**]), 1 phloretin glucoside (phlorizin [**30**]) and 2 isomers of a tetrahydroxyflavanonol-O-hexoside ([**25** and **29**]). From these compounds, epicatechin gallate (**27**) and phlorizin (**30**) were previously detected in acetone extract from *Persicaria* (syn. *Polygonum*) *hydropiper* cell suspension cultures (Ono et al., 1998) and in *Fallopia japonica* (syn. *P. cuspidatum*) flowers methanol extract (Sun et al., 2014), respectively. Quercetin-galloylgalactoside (**26**) was also isolated before from *P. viscosum* Buch.-Ham. Ex D. Don methanol extract (Datta et al., 2000). In addition, different proanthocyanidins were found in 90% methanol extracts from *Fallopia multiflora* (syn. *P. multiflorum*) stems (Wang et al., 2013), as well as in 96% ethanol extract from *P. coriarium* roots (Makhmatkulov et al., 1992).

3.3. In vitro skin care properties

The phenolic enriched extract of *P. maritimum* was evaluated for *in vitro* antioxidant properties through four complementary methods, namely total antioxidant (TAC), superoxide ($O_2^{-\cdot}$) radical-scavenging and initial (FTC) and late (TBA) lipid peroxidation activities. Results are shown in Table 4. The extract showed a strong antioxidant activity, and it was particularly effective as an O_2-· scavenger, with an IC_{50} value of 40.4 µg/mL, significantly lower ($p < 0.05$) than that of positive control (catechin: IC_{50} = 61.6 µg/mL). It also had a significant transition metal ion reducing – antioxidant capacity in the phosphomolybdenum assay (TAC; IC_{50} = 647 µg/mL) and in the late lipid peroxidation assay

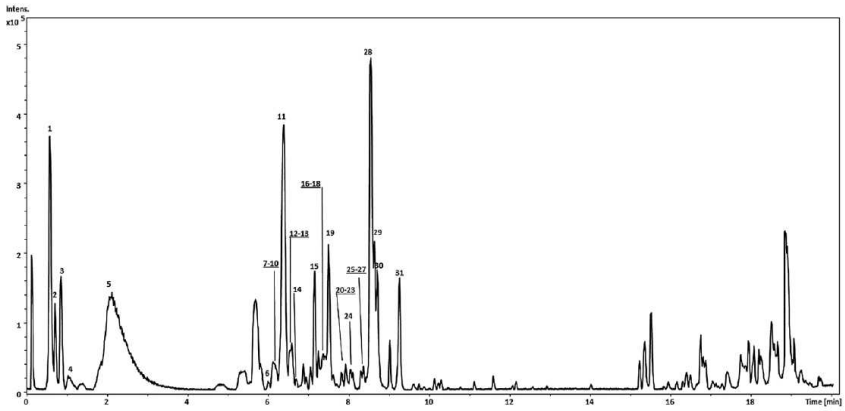

Fig. 1. UHRMS analysis of the acetone extract of sea knotgrass (*P. maritimum*) aerial parts. Peak numbers refer to the compounds listed in Table 3.

Table 4
Relevant biological activities of the acetone extract of sea knotgrass (*P. maritimum*) aerial parts. Results are expressed as IC_{50} values (μg/mL).

Assay	Extract	Positive Control
Antioxidant activity		
RSA of $O_2^{-\cdot}$	40.4 ± 2.4[a]	61.6 ± 4.9[b]
TBA	784 ± 35[b]	92.9 ± 0.1[a]
TAC	647 ± 5[b]	130 ± 1[a]
Anti-inflammatory activity		
Reduction of NO production	22.0 ± 2.6[a]	27.6 ± 1.0[a]
Anti-melanogenic activity		
TYRO inhibition	64.1 ± 0.3[a]	137 ± 6[b]
Reduction of melanin production	77.7 ± 2.6[b]	16.0 ± 0.5[a]

Values represent the mean ± standard error of the mean (SEM) of at least three experiments each performed in triplicate (n = 9). In the same line, values followed by different letters are significantly different at $P < 0.05$ (student's *t*-test). Positive controls: RSA of $O_2^{-\cdot}$ (catechin), TBA (BHT), TAC (ascorbic acid), reduction of NO production (L-NAME), TYRO inhibition and reduction of melanin production (arbutin).

(TBA; IC_{50} = 784 μg/mL). However, the extract did not show significant activity on early lipid peroxidation (FTC) up to 10,000 μg/mL. Methanol and dichloromethane extracts from aerial parts of this species have already displayed strong antioxidant properties (El-Haci et al., 2013; Rodrigues et al., 2017), as well as from *P. sachalinensis* F.Schmidt and *P. cuspidatum* (Pan et al., 2007; Fan et al., 2011)

Oxidative stress increases with ageing, while the endogenous defence potential decreases, which leads to progressive impairment of cell functions. Skin cells are rich in polyunsaturated fatty acids that are particularly susceptible to oxidative damage that contributes to wrinkles formation, photoaging, elastosis, dry skin and hyperpigmentation (Bogdan Allemann and Baumann, 2008; Kusumawati and Indrayanto, 2013). Topical antioxidants may reduce or inhibit the oxidation reactions and thus, protect the skin from the environmental stress caused by free radicals (Kusumawati and Indrayanto, 2013). In addition, antioxidants also have a crucial role in safety and quality of final formulation by increasing the product shelf life (Jung et al., 2017). Many commercial cosmetic products include combinations of different plant extracts rich in natural antioxidants with these purposes. For example polyphenols, including flavonoids (catechins and myricetin derivatives), as those found in *P. maritimum* acetone extract, are described with strong antioxidant properties (Rice-Evans et al., 1996). Our results indicate that *P. maritimum* extract has strong antioxidant ability and that it has the potential to be used as cosmetic ingredient for prevention oxidative stress in the skin.

Lipopolysaccharide (LPS) is a cell wall endotoxin from Gram-negative bacteria that induce the production of inflammatory mediators, such as NO, by activated macrophages. Thus, reduction in *in vitro* NO production by macrophages is used as an anti-inflammatory model (Rodrigues et al., 2014, 2017). Following this, the selected sea knotgrass extract was evaluated for its potential anti-inflammatory properties. The extract was first tested for its toxicity (Fig. 2) on RAW 264.7 macrophages, showing a slight toxicity at 100 μg/mL (27% of cellular viability), however from 50 to 3.125 μg/mL the extract did not showed any toxicity, exhibiting cellular viabilities higher than 95% (Fig. 2). Afterwards, the nontoxic concentrations of the extract were tested for the NO production assay; the sea knotgrass acetone extract showed a strong reduction of NO production, with an IC_{50} value of 22 μg/mL, similar to the positive control (L-NAME; IC_{50} = 27.3 μg/mL; Table 4). This result agrees with previous reports describing the anti-inflammatory activity of a dichloromethane leaf extract of this species (IC_{50} = 48 μg/mL; Rodrigues et al., 2017). Moreover, other *Polygonum* species, such as *P. lapathifolium* L. and *P. perfoliatum* L. were already described with the same properties (Takasaki et al., 2001; Kim et al., 2007; Lei et al., 2015).

Inflammation is involved in skin ageing process. The presence of an infectious agent or damaged tissue induces an inflammatory response, through the production of inflammatory mediators (cytokines) by immune cells, such as leucocytes and macrophages, which also induces the production of reactive oxygen (ROS) and/or nitrogen species (Bennett et al., 2008; Rinnerthaler et al., 2015). Moreover, ROS and cytokines increase the production of proteolytic enzymes that degrade the extracellular matrix, decreasing skin elasticity and increasing wrinkles formation. Inflammation caused by environmental conditions (*e.g.* pollution), UV photodamage, or infection (*e.g.* acne vulgaris) results in skin aging and skin abnormalities, including flushing and irritation (Gragnani et al., 2014; Mistry, 2017). Anti-inflammatory agents, such as phenolic compounds, are commonly incorporated into skin care products to improve skin tone and texture by preventing the collagen and elastin degradation and increasing fibroblastic synthesis of collagen (Mistry, 2017). For example, the main compounds found in the acetone extract of sea knotgrass were previously described with anti-inflammatory properties, namely the flavonoids myricitrin (**28**) (Domitrović et al., 2015) and catechin (**11**), as well as monogalloylhexose (**1**) that has a similar structure to β-glucogallin, known for its capacity to reduce LPS-induced inflammation in murine macrophages (Chang et al., 2013). Moreover, other flavonoids found in this extract can contribute to this capacity, such as other catechins and proanthocyanidins that are also described with anti-inflammatory properties (Rathee et al., 2009; Nichols and Katiyar, 2010). Our results suggest that this extract has anti-inflammatory properties, with interest in the prevention of skin inflammatory problems related with pollution, UV radiation and/or acne vulgaris.

To assess the *in vitro* anti-melanogenic activity, the sea knotgrass extract was tested against tyrosinase, a copper-containing metalloprotein enzyme involved in melanin biosynthesis. Table 4 shows that the acetone extract of *P. maritimum* inhibited tyrosinase (IC_{50} = 64.1 μg/mL) significantly stronger than that obtained with the standard (arbutin; IC_{50} = 137 μg/mL). To further explore the anti-melanogenic

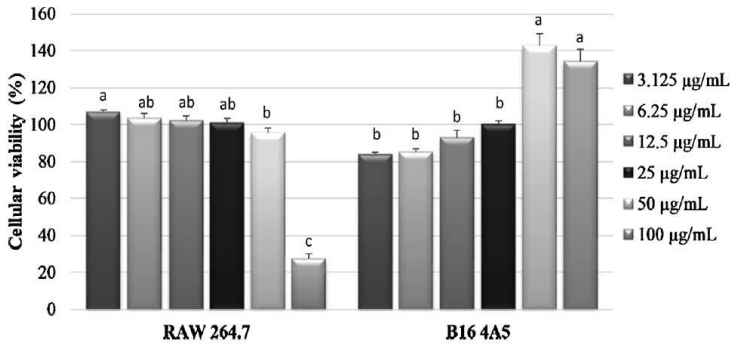

Fig. 2. Toxicity of the acetone extract of sea knotgrass (*P. maritimum*) aerial parts on RAW 264.7 macrophages and B16 4A5 melanoma cell lines. Results are expressed as a percentage (%) of cellular viability relative to a control with the vehicle (0.5% DMSO, v/v).

properties of the extract, a cell model of melanogenesis was used (B16 4A5 melanoma cells). To identify the nontoxic concentrations of the extracts to be used in the assay, samples were tested for its toxicity on B16 4A5 melanoma cells (Fig. 2). No significant toxicity was obtained after application of the samples up to concentrations of 100 µg/mL (Fig. 2), and all the tested concentrations were evaluated for their effect on melanin production. The sea knotgrass acetone extract showed a high ability to reduce melanin production on the B16 4A5 melanoma cellular model (IC_{50} = 77.7 µg/mL). As far as we know, this is the first report on the anti-melanogenic properties of *P. maritimum*. However, other species from the same family (Polygonaceae), such as a dichloromethane extract of stem and leaves of *Ruprechtia* sp., displayed a significant anti-tyrosinase activity (IC_{50} = 33.76 µg/mL; Macrini et al., 2009). An acetone extract of the rhizomes of *Rheum officinale* Baillon also showed inhibition against tyrosinase along with inhibition of melanin biosynthesis on B16 cells (Iida et al., 1995).

Despite the melanin role in skin protection against UV radiation, its overproduction and accumulation can lead to high pigmented patches and/or discolorations, namely melasma, freckles, post-inflammatory melanoderma and solar lentigo (Tu and Tawata, 2014). As stated previously, the melanin biosynthesis is modulated by tyrosinase, which is an important regulator and rate-limiting enzyme for the melanogenic process (Tu and Tawata, 2014). In this sense, melanin synthesis and tyrosinase inhibitors have extensive cosmetic applications as skin-whitening products to treat excessive accumulation of melanin (Hakozaki et al., 2017). For example, phenolic compounds have protective effects on UV-mediated skin pigmentation, due to their antioxidant and UV-absorbing actions (Chaiprasongsuk et al., 2016). Catechins and its diverse derivatives, which are present in high amounts in *P. maritimum* acetone extract, were reported with strong depigmenting effects on B16 melanoma cells through the reduction of tyrosinase expression (Sato and Toriyama, 2009). Furthermore, β-glucogallin, a compound structurally similar to monogalloyl-hexose (**1**), the third major compound identified in the sea knotgrass extract, has already been demonstrated to reduce cellular melanin concentration B16F1 mouse melanoma cells (Majeed et al., 2010). Also, proanthocyanins and its dimers, also found is the extract, are described to reduce the melanogenesis (Tatsuno et al., 2012). Thus, the results obtained with the sea knotgrass extracts indicate it is a good source of phenolic compounds for potential application in cosmetic products for skin hyperpigmentation.

The *in vitro* anti-wrinkles properties of the sea knotgrass extracts were evaluated through elastase inhibitory capacity, but no significant activity was observed after the application of the different concentrations of the extract, although different *Polygonum* species have already shown anti-wrinkles activity. For example, a cosmetic formulation containing a water extract from the seeds of *P. minus* had anti-wrinkles activity in clinical trials (Haris et al., 2014). A 70% ethanol extract made from flowers of *P. tinctorium* had *in vitro* anti-elastase activity (Kang et al., 2017), whereas *P. multiflorum* (*Fallopia multiflora*) root aqueous extract had both *in vitro* anti-elastase and -collagenase capacities (Kim and Leem, 2014). It could be that the anti-wrinkle properties are due to highly polar compounds that are not (or poorly) extracted with acetone in our study. The ability of the sea knotgrass acetone extract as anti-acne was also assessed, through the inhibition of lipase. The extract did not show anyshow significant anti-lipase activity up to 10,000 µg/mL. However, other species from the Polygonaceae family have already been reported with lipase inhibitory activity, namely *Rheum palmatum* L., *R. ribes* and *Fallopia japonica* methanol extracts (Seyedan et al., 2015). Additionally, the antimicrobial potential of *P. maritimum* acetone extract was evaluated against different microorganisms, including *E. coli, S. aureus, P. aeruginosa, C. albicans*. However, no significant activity was found, although methanol extracts from this species was previously described with remarkable antibacterial activity against *S. aureus* (MIC = 0.12 mg/mL), *P. aeruginosa* (MIC = 64.35 mg/mL) and *E. coli* (MIC = 16.08 mg/mL) (El-Haci et al., 2013). Here again, it could be that the antimicrobial activity is due to highly compounds not (or poorly) extracted with acetone. Besides, since this preliminary work was done only with the raw extract, the presence of some anti-elastase, anti-lipase and antimicrobial compounds in the sea knotgrass could not be excluded and further investigations in the purified extract is needed before conclusions could be drawn.

4. Conclusions

Sea knotgrass is a rich source of phenolics, particularly flavonoids, and acetone was the most efficient solvent in extracting these compounds, when using a 30 min ultrasonic bath, which doubled the extraction yield. The sea knotgrass phenolic-enriched extract was an effective $O_2^{-\cdot}$ scavenger, showed potent antioxidant capacity on the phosphomolybdenum assay (TAC), and strong inhibition of late lipid peroxidation assay (TBA). Moreover, it showed a high anti-inflammatory potential in the reduction of NO production on LPS-stimulated RAW 264.7 macrophages, as well as high *in vitro* anti-melanogenic activity through tyrosinase inhibition and reduction of melanin production by B16 melanoma cells. The sea knotgrass acetone extract was nontoxic at the active concentrations on both cell lines. However, the extract did not display significant inhibition of initial lipid peroxidation (FTC), nor anti-wrinkles (elastase), anti-acne (lipase), or antimicrobial activities (*E. coli, S. aureus, P. aeruginosa, C. albicans*). Additionally, this species showed high contents of phenolics, including 24 flavonoids, 5 phenolic acids and 1 acetophenone that were identified by UHRMS analysis. Among these, myricitrin, catechin and monogalloyl-hexose were the major components detected, and have recognized health promoting properties, namely as antioxidant, anti-inflammatory and anti-melanogenic properties with potential skincare applications. These data indicate that the aerial parts of *P. maritimum* are a promising source of high value-added ingredients with skincare properties for the cosmetic industry, namely antioxidant, anti-inflammatory and anti-melanogenic activities. To guarantee the supply to the industry, assays regarding the sustainable production of *P. maritimum* in greenhouse are already being pursued.

Conflict of interest

The authors declare that they have no conflict of interest.

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The irrigation salinity and harvesting affect the growth, chemical profile and biological activities of *Polygonum maritimum* L.

Maria João Rodrigues[a], Ivo Monteiro[b], Chloé Placines[a], Viana Castañeda-Loaiza[a], Sylwester Ślusarczyk[c], Adam Matkowski[c], Catarina Pereira[a], Pedro Pousão-Ferreira[b], Luísa Custódio[a,*]

[a] Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
[b] IPMA, Aquaculture Research Station, Olhão, Portugal
[c] Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Wroclaw, Poland

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ABSTRACT

Previously, our group identified the halophyte *Polygonum maritimum* L. (sea knotgrass) as a promising source of ingredients for the cosmetic, food, pharmaceutical and veterinarian industries. To further explore this species, and to guarantee the supply of biomass for commercial purposes, it is necessary to ensure its sustainable production while assuring the preservation of its chemical and biological properties. In this context, this work aimed to cultivate this species in greenhouse conditions and to determine the influence of specific agronomic conditions, namely irrigation salinity and harvest, on the plant's growth performance and biological properties of obtained biomass. For that purpose, plants were grown in a greenhouse and irrigated with water with different salinities (0, 100, 200, 300 and 600 mM of sodium chloride - NaCl). After six weeks of irrigation, plants were cut 7 cm above soil level and submitted to an additional two harvests with a six-week interval. Plant growth performance was evaluated in terms of plant height, leaf number and surface area, moisture, and productivity. Acetone extracts were prepared from aboveground organs and evaluated for chemical composition (by spectrophotometric methods, and by ultra-high-resolution mass spectrometry - UHRMS), and for *in vitro* antioxidant properties [radical-scavenging activity (RSA) on DPPH and ABTS, ferric reducing antioxidant power (FRAP) and metal chelating activity on iron (ICA) and copper (CCA)]. Extracts were also appraised for *in vitro* anti-inflammatory activity on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Increased salinity and multiple harvests reduced plant growth and yields; the highest productivity was obtained in plants irrigated with freshwater at 2nd harvest (346 g/m^2 of dried biomass). The extracts maintained the *in vitro* biological properties and interesting chemical profile, however, these depended on the irrigation salinity and harvest regime. The highest antioxidant activities were obtained in extracts from plants irrigated with freshwater at the 3rd harvest (DPPH: 96.2%; ABTS: 89.1%; CCA: 61.6%; FRAP: 136%). The extracts from biomass obtained in the 1st harvest of freshwater-irrigated plants, and from those treated with 100 and 200 mM of NaCl, had significant anti-inflammatory properties. The main compounds detected were mostly flavonols (myricetin and quercetin glycosides), which varied according to both irrigation salinity and harvest. Our results indicate that sea knotgrass can be cultivated in greenhouse conditions aiming industrial commercial applications, irrigated with freshwater or with irrigation solutions with moderate salinity. Moreover, produced biomass maintain the biological and chemical properties previously detected in plants collected from the wild.

1. Introduction

Halophyte plants are the flora of saline environments, are abundant in coastal areas worldwide and can endure saline soils containing up to 1 M NaCl (*e.g. Tecticornia* spp; English and Colmer, 2013). The classification of halophytes regarding their salt tolerance is still highly debated (Grigore et al., 2012). Irrespective of the degree of salt tolerance, these plants can be classified into different categories concerning their ecological behaviour, distribution, response to salinity and salt intake (Aslam et al., 2011). In respect to the salt intake, and according

* Corresponding author at: Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal.
E-mail address: lcustodio@ualg.pt (L. Custódio).

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to Aslam et al. (2011), halophytes can be grouped into obligate (also called true halophytes, includes members of the family Chenopodiaceae) that require salt to achieve optimal growth rates; facultative (members of Gramineae, Cyperaceae and Juncaceae families), which exhibits optimum growth in saline conditions,but are also able to grown in non-saline environments; or habitat-indifferent (*e.g. Juncus bufonius* L.), with optimum growth occurring in non saline conditions, but that are also able to complete their life cycle in saline environments. Halophytes can also be categorized according to their different adaptations to salinity through two main mechanisms, namely salt tolerance (maintenance of cytoplasmic homeostasis, for example *Spartina* sp. and *Distichlis* sp.; Ksouri et al., 2012; Aslam et al., 2011; Meng et al., 2018), and salt avoidance (through the prevention of salt accumulation by decreasing the salt concentrations inside of the cells, for example, *Mesembryanthemum crystallinum* and *Salicornia* sp.; Aslam et al., 2011; Meng et al., 2018). A detailed discussion of the mechanisms underlying the salt tolerance in halophytes can be found elsewhere (*e.g.* Flowers and Colmer, 2008; Flowers et al., 2010; Ali and Yun, 2017; Mishra and Tanna, 2017).

Besides salinity, the halophyte´s habitat is characterized by other stressful abiotic constraints, such as drought and high ultraviolet (UV)-radiation (Koyro and Huchzermeyer, 2004; Flowers and Colmer, 2008). To withstand these conditions, and salinity, these plants have developed diverse physiological and biochemical adaptations, which include antioxidant enzymatic defence mechanisms (*e.g.* glutathione reductase, ascorbate peroxidase (APX), peroxidase (POX) and catalase; Boestfleisch et al., 2014), and the synthesis and accumulation of different primary and secondary metabolites, for example, fatty acids, phenolics, alkaloids and phytosterols (Ksouri et al., 2012). Besides their plant protective properties, the latter molecules have important biological properties such as antioxidant, anti-inflammatory, antidiabetic and neuroprotective (Flowers et al., 2010; Ksouri et al., 2012), which may explain the several ethnomedicinal and -veterinary uses of different halophyte species (Ksouri et al., 2012). For example, *Salicornia* L. species (sea asparagus) are used in traditional medicine against obesity and diabetes while *Crithmum maritimum* L. (sea fennel) is used as diuretic and antiscorbutic (Pereira et al., 2017).

Despite the recent interest on the commercial potential of halophytes (Ksouri et al., 2012; Petropoulos et al., 2018a, b) knowledge about the cultivation of such species is scarce, and there is a need to develop production systems for target species that are economically, socially and environmentally feasible. Halophytes can be cultivated in conditions where commercial glycophytes cannot; while the productivity of salt-sensitive species (glycophytes) is highly affected in salt-affected soils, the growth of several halophytic species is stimulated within a salinity range of 15–25 dS. Halophytes can be commercially produced, for example, in greenhouse irrigated with saline water (*e.g. Salicornia* sp. in RiaFresh [Portugal] and Koppert Cress [Netherlands]) or in saline soils (*e.g.* sea asparagus and *Inula crithmoides* L. [golden samphire] in Horta Salgada, Portugal). Halophytes can also be farmed *via* integrated multi-trophic aquaculture (IMTA) systems, where the by-products (wastes) from one or more marine organisms' species (*e.g.* fish) are recycled to become fertilizers for plant cultivation. For example, there are reports of the successful growth of *S. europaea* with effluents from a shrimp, sole, and turbot farming, and of *Tripolium pannonicum* (Jacq.) Dobrocz., *Plantago coronopus* L., (buckshorn plantain) and *S. dolichostachya* (Moss.) coupled with the production of European sea bass (*Dicentrarchus labrax* L.) (Waller et al., 2015). Moreover, the levels of secondary metabolites responsible for the medicinal properties of halophytes may be influenced by agrotechnical practices, as for example, quantity and salinity of irrigation water, fertilization, harvest time and cycle (Ventura et al., 2010, 2011, 2013).

There are some studies describing the influence of saline conditions on the growth and biochemical properties of different halophyte species, including on nutritional profile (Glenn et al., 1991; Zurayk and Baalbaki, 1996; He et al., 2003; Rao et al., 2004; Koyro, 2006; Ventura et al., 2011; Pearlstein et al., 2012; Belkheiri and Mulas, 2013; Díaz et al., 2013; Shpigel et al., 2013; Benzarti et al., 2014; Waller et al., 2015). However, there is a significantly lower number of studies related with the influence of salinity on the biological activities of obtained biomass, including on antioxidant properties (Ksouri et al., 2007; Boestfleisch et al., 2014; Slama et al., 2015; Bendaly et al., 2016; Slama et al., 2017). In our ongoing efforts aiming a smart commercial valorisation of halophytic biodiversity in southern Portugal, we have identified *Polygonum maritimum* L. (common name: sea knotgrass), as a rich source of bioactive phenolic compounds, particularly flavonoids, and of natural products with *in vitro* antioxidant, anti-inflammatory, antimicrobial, antidiabetic, neuroprotective and anti-melanogenic properties (El-Haci et al., 2013; Rodrigues et al., 2017, 2018, 2019). To further explore this species, and to guarantee the supply of biomass for commercial purposes, it is necessary to ensure its sustainable production while assuring the preservation of its chemical and biological properties. In this context, in this work, sea knotgrass was produced in greenhouse conditions and irrigated with irrigation solutions with different salinity levels, ranging from 0 (freshwater) until 600 mM NaCl. The growth performance was evaluated in terms of plant height, leaf number and surface area, biomass production and yields (g/m^2 of fresh biomass). Acetone extracts were prepared from aerial organs and evaluated for functional properties (*in vitro* antioxidant and anti-inflammatory activities), and for chemical composition by spectrophotometric methods and ultra-high-resolution mass spectrometry (UHRMS) analysis.

2. Materials and methods

2.1. Chemicals, culture media and supplements

The 1,1-diphenyl-2picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), sodium nitrite, lipopolysaccharide (LPS) from *Escherichia coli*, sulphanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride (NED), catechin, and rutin were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied Folin-Ciocalteau (F-C) phenol reagent and all solvents used for chemical analysis. Other reagents and solvents were acquired from VWR International (Belgium). The murine RAW 264.7 leukemic monocyte-macrophage cell line was obtained from the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal).

2.2. Germination

Sea knotgrass seeds were bought from "Sementes de Portugal" (Caldas da Rainha, Portugal). For germination optimization, four groups of 20 seeds each were subjected to different treatments: 1) control – no treatment, 2) overnight freshwater immersion, 3) scarification, and 4) scarification followed by overnight freshwater immersion. After treatments, seeds were potted in polystyrene plant trays (1 seed per each 3 x 3 cm alveoli), in a 3:1 mixture of peat and perlite (v/v). Germinated seeds were counted daily for 4 weeks. Germination and subsequent cultivation were performed in plastic-greenhouse conditions. The minimum and maximal temperature and relative humidity of the greenhouse during the experiments are summarized in Fig. S1.

2.3. Plant culture

Ten weeks after germination initiation, the plants (15 per treatment) were transplanted to 7 × 7 x 15 cm plastic plant trays in 1:1 mixture of peat + perlite (3:1, v/v) and washed beach sand. After two weeks of plant adaptation (irrigated with freshwater), plants were irrigated with progressively increasing concentrations of artificial saltwater, starting from 50 mM with an increase of 50 mM every two days up to the final concentration. Plants were watered every two days with

100 mL of the irrigation solutions with different NaCl levels, namely approximately 0 (freshwater), 100, 200, 300 and 600 mM, by each alveolus. Once a week the irrigation solutions were supplemented with liquid fertilizer (NPK 7-5-6). After 6 weeks of saline irrigation, plants were cut approximately 7 cm above soil level (minimum of 20 cm height). Plants were submitted to additional two harvests with a 6-week interval. The photoperiod varied among 14/10 h and 13/11 h (day/night) for 1–14 and 15–18 weeks, respectively. The greenhouse temperature and relative humidity conditions, during the 18 weeks of the treatments, are presented in Fig. S1 of supplementary material. To chemical and functional properties determination, samples from identical conditions were pooled in a single sample, freeze-dried, powdered and stored at -20 °C.

2.4. Growth performance and moisture contents

Before harvesting, the total plant height and leaf number were recorded, and after harvesting, the leaf surface area was determined (10 leaves per treatment). Fresh (FW) and dry (DW) weights of aerial parts were determined and the moisture contents calculated by the difference between FW and DW. Plant survival and biomass production (g/m^2 of dried biomass) were also determined.

2.5. Extraction

Ultrasound-assisted extraction was used for the preparation of acetone extracts from the dried biomass of aerial parts, containing leaves, stems and shoots. Samples were mixed with 100% acetone (1:40, w/v) and extracted in an ultrasonic bath for 30 min (Rodrigues et al., 2019). The extracts were filtered (Whatman no 4) to remove solid debris, acetone was removed by using a rotary evaporator, obtained extracts were weighed and dissolved in methanol at 10 mg/mL and stored at -20 °C.

2.6. Antioxidant activity

2.6.1. Radical-scavenging activity (RSA) on DPPH˙ and $ABTS^{+\cdot}$

Samples were tested for RSA against the DPPH and ABTS radicals at concentrations ranging from 10 to 1000 µg/mL, as described previously (Rodrigues et al., 2015). Butylated hydroxytoluene (BHT) was used as a positive control at the same concentrations of the samples. Results were expressed as a percentage of inhibition, relative to a control containing methanol in place of the sample, and when possible, as half maximal inhibitory concentration (IC_{50} values, µg/mL).

2.6.2. Ferric reducing antioxidant power (FRAP)

The ability of the extracts (at concentrations ranging between 10–1000 µg/mL) to reduce Fe^{3+} 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, 1000 µg/mL), and as IC_{50} values (µg/mL).

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

ICA and CCA were tested on samples at different concentrations (10–1000 µg/mL) as described previously (Rodrigues et al., 2015). The change in colour was measured on a microplate reader (Biotek Synergy 4), and ethylenediaminetetraacetic acid (EDTA) was used as the positive control at the same concentrations of the samples. Results were expressed as a percentage of inhibition, relative to a control containing ultrapure water in place of the sample, and as IC_{50} values (µg/mL).

2.7. In vitro anti-inflammatory activity on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages

2.7.1. Cell culture and cell viability

RAW 264.7 macrophages were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL) / streptomycin (50 µg/mL) and were maintained at 37 °C in humidified atmosphere with 5% CO_2. Exponentially growing cells were plated in 96-well tissue plates at a density of 1×10^4 cells/well, followed by 24 h of incubation. Samples were then applied at different concentrations (3–100 µg/mL) for 24 h. Control cells were treated with DMSO at the highest concentration used in test wells (0.5%), and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as described previously (Rodrigues et al., 2014). Results were expressed as the percentage of viability, in comparison with the control cells.

2.7.2. In vitro anti-inflammatory assay

The effect of the application of the extracts on the nitric oxide (NO) production was evaluated in RAW 264.7 macrophages (Rodrigues et al. (2014). Cells were seeded at 2.5×10^5 cells/well in 96-well tissue plates, left to adhere overnight and treated with non-toxic concentrations of the samples (those that resulted in cellular viabilities greater than 80%), in serum- and phenol-free culture medium, containing 100 ng/mL of LPS, for 24 h. The NO production by macrophages was assessed by the Griess assay (Rodrigues et al., 2014). Results were expressed as a percentage (%) of NO production, relative to control treated with culture medium supplemented with DMSO (0.5%, v/v), and when possible as IC_{50} values (µg/mL).

2.8. Chemical composition

2.8.1. Determination of total phenolic (TPC) and flavonoid (TFC) contents

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

2.8.2. Ultra-high-resolution mass spectrometry (UHRMS)

The extracts (100 mg) were dissolved in 1 mL of ultra-pure water (HLP10Uv, Hydrolab, Gdańsk) acidified with 0.2% formic acid and purified by Solid Phase Extraction (SPE) using C18 Sep-Pak cartridges (1 cm^3, 360 mg, Waters Corp., Milford, MA). The cartridges were washed with 0.5% methanol to remove carbohydrates and then with 80% methanol to elute phenolics. The phenolic fraction was re-evaporated and dissolved in 1 mL of 80% methanol (acidified with 0.2% formic acid). The sample (intense yellow colour without chlorophyll) was then centrifuged (23 000 x g, 5 min) and filtered (0.22 µm) before LC–MS analysis. All analyses were performed in triplicate for three independent samples (stored at -20 °C before analysis for no longer than 3 days).

Liquid chromatography (LC) – electrospray ionization (ESI)-QTOF-MS estimation of polyphenol composition was carried out on a Thermo Dionex Ultimate 3000 RS (Thermo Fischer Scientific, Waltham, MA) chromatographic system, coupled to a Bruker Compact (Bruker, Billerica, MA) quadrupole time-of-flight (QTOF) mass spectrometer, consisting of a binary pump system, sample manager, column manager and PDA detector. Separations were performed on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, USA), with mobile phase A consisting of 0.1% (v/v) formic acid in water and mobile phase B consisting of 0.1% (v/v) formic acid in acetonitrile. A linear gradient

from 1% to 60% phase B in phase A over 20 min was used to separate phenolic compounds. The flow rate was 0.4 mL/min, and the column was held at 30 °C. Spectra were acquired in negative-ion mode over a mass range from *m/z* 100 to 1500 with 5 Hz frequency. Operating parameters of the ESI ion source were as follows: capillary voltage 3 kV, dry gas flow 6 L/min, dry gas temperature 200 °C, nebulizer pressure 0.7 bar, collision radio frequency 700.0 V, transfer time 100.0 µs, and pre-pulse storage 7.0 µs. Ultrapure nitrogen was used as drying and nebulizer gas, and argon was used as the collision gas. The collision energy was set automatically from 15 to 75 eV depending on the *m/z* of the fragmented ion. Acquired data were calibrated internally with sodium formate introduced to the ion source at the beginning and end of each separation *via* a 20 µL loop. Processing of spectra was performed with Bruker DataAnalysis 4.3 software. The quality of the isotopic fit was expressed by the mSigma-value. The matched peaks from SmartFormula3D were sent to MetFrag website for computer-assisted *in silico* fragmentation and identification of metabolite mass spectra. Additionally, web-based databases were used to search for the identity of the detected compounds: the human metabolome database (http://www.hmdb.ca/), the BiGG database (http://bigg.ucsd.edu/), the PubChem database (http://pubchem.ncbi.nlm.nih.gov/), the MassBank database (http://www.massbank.jp), KEGG (www.genome.jp) and the Metlin database (http://metlin.scripps.edu).

After data acquisition, raw UPLC – QTOF-MS spectra (negative mode) were pre-processed using a ProfileAnalysis software (version 2.1, Bruker Daltonik GmbH, Germany). Parameters of ProfileAnalysis were used as follows: advanced bucket generation with retention time range of 0–20 min, mass range of 100–800 *m/z*, each bucket (spectral bins) was formed with 1 min and 1 *m/z* delta, 0.2 kernelizing value, without normalization, background subtraction, and time alignment. LC–MS analyses were processed with the Find Molecular Futures (FMF) function to create compounds (molecular features) with S/N- 3 for peak detection. Generated bucket table consisting of t_R:*m/z* pairs and respective compound intensity was exported and uploaded to MetaboAnalyst program. Each obtained dataset was filtered and normalized to the sum of peak areas and mean-centered scaling. The multivariate PCA and PLS-DA methods were used to investigate the overall variation in the metabolome for each experimental group (0, 100, 200 and 300 mM of NaCl).

2.9. Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM), and experiments were led at least in triplicate. Significant differences were evaluated by analysis of variance (ANOVA) and by the Tukey HSD test ($P < 0.05$). Statistical analyses were made using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation). The IC_{50} values were calculated by a sigmoidal fitting of the data (GraphPad Prism v. 5.0 program).

3. Results and discussion

3.1. Plant growth performance

The germination of halophyte plants is influenced by several factors, as for example salinity and temperature (Khan et al., 2003). For many species, the best germination rates are attained with freshwater (Ungar, 1991), as for example, for *Salicornia* spp. (Ventura et al., 2011), and usually, seeds do not withstand high salinity conditions, during germination (Khan et al., 2006). In this work, four germination treatments were performed on sea knotgrass seeds, namely overnight immersion in freshwater, scarification, and scarification followed by overnight freshwater water immersion. Germination was carried out in plastic-greenhouse conditions (for the conditions during the greenhouse experiments please see Fig. S1). The first seeds germinated after 13 days, for all treatments, except for the scarification treatment, followed by overnight water immersion that took 18 days to germinate (data not shown). At the end of the 4th week, we observed a germination percentage of 30, 25, 10 and 15%, in the sequence mentioned above (data not shown). In a previous work, the germination rate of *P. maritimum* seeds collected from the Atlantic seaboard of northern Spain was highest at 30/20 °C (day/night), but cold stratification (12 weeks at 3 °C) reduced the optimum temperature range to 14/4 °C (day/night) (Fernandez-Pascual et al., 2017). Moreover, it is known that newly formed seeds from some *Polygonum* species, as for example *P. aviculare*, are dormant (Khan and Ugar, 1998). Therefore, different strategies can be tested in order to improve the germination of *P. maritimum*, including for example, temperature, cold stratification and gibberelic acid treatment (Khan and Ungar, 1998; Sadeghi and Rasouli, 2012).

Although it is accepted that halophyte plants require salt to complete their life cycle, some studies on the halophytes' cultivation showed that many of these species have better growth rates in the absence of salt (Grigore et al., 2012). Thus, after transplantation and acclimatization, plantlets were submitted to different irrigation treatments, including approximately 0 (freshwater), 100, 200, 300 and 600 mM of NaCl (prepared in freshwater). After 6 weeks of the irrigation treatments, plants were cut approximately 7 cm above soil level (1st harvest), and 2 additional cuts were made with a 6-week interval. All plants survived to all irrigation treatments until the 1st harvest, however only the plants subjected to irrigation with freshwater (0 NaCl), 100 mM and 200 mM treatments survived until the 2nd cut, as well as the plants from control and 100 mM treatments that survived up to the final cut (data not shown). Thus, from now on, results will be only presented for the conditions that allowed the survival of the plants.

Sea knotgrass is considered a glycohalophyte, which means that its salt protection mechanism is salt avoidance (salt-impermeable), being dependent on the synthesis of organic osmolytes, namely sugars and amino acids, which have a high maintenance cost to keep osmotic balance (Guy et al., 1988; Kosakivska et al., 2017). Thus, due to the severe requirements to survive under extreme salinity stress, the plant mortality increases with growing salinity on the growth medium, as observed in our experiment where plants did not survive to irrigation with higher levels of NaCl (van Puijenbroek et al., 2017).

Fig. 1 shows the growth performance parameters of sea knotgrass plants for each harvest and salinity irrigation treatment. The plant height was similar between different salinity irrigation treatments for the 1st harvest (29.5–35 cm), except for 300 mM, where it was lower (17.6 cm); however, it decreased significantly in the 2nd and 3rd harvests (12.9–16.5 cm), except for plants irrigated with freshwater (29.5–34 cm; Fig. 1A). Regarding the leaf number, on freshwater-irrigated plants it increased in the 2nd and 3rd harvests (from 61.1 to 85.5 and 91.4, respectively), but remained similar for amongst all cuts for 100 mM (45.4–52.7); however for the 200 mM treatment, it diminished in the 2nd cut (from 49 to 24.7), as well as the 1st harvest of 300 mM (18.9; Fig. 1B). The results for the leaf surface area showed an opposite tendency amid the different irrigations: it decreased between harvests in the same treatment and reduced with increased salinity amongst the different irrigations (Fig. 1C). The moisture level decreased with the harvests and with the increasing salinity treatments, and the 1st harvest of freshwater-irrigated control plants showed the highest content (79.9%), while the 1st harvest of 300 mM had the lowest level (55.8%; Fig. 1D). When compared with saline irrigation conditions, the productivity was higher for the plants irrigated with freshwater (201–346 g/m^2 of dried biomass), and the highest value was obtained on the 2nd harvest. Besides, the productivity diminished with the growing irrigation salinity, and amongst harvest for the same irrigation conditions (Fig. 1E).

All these data agree with that obtained by Grigore et al. (2012), that observed that after 12 weeks of cultivation of the halophytes *Inula crithmoides* L., *Plantago crassifolia* Forssk., and *Medicago marina* L., the plants grown in salt absence had better productivity and growth rates, thus suggesting that at least some halophytes can grow well in non-

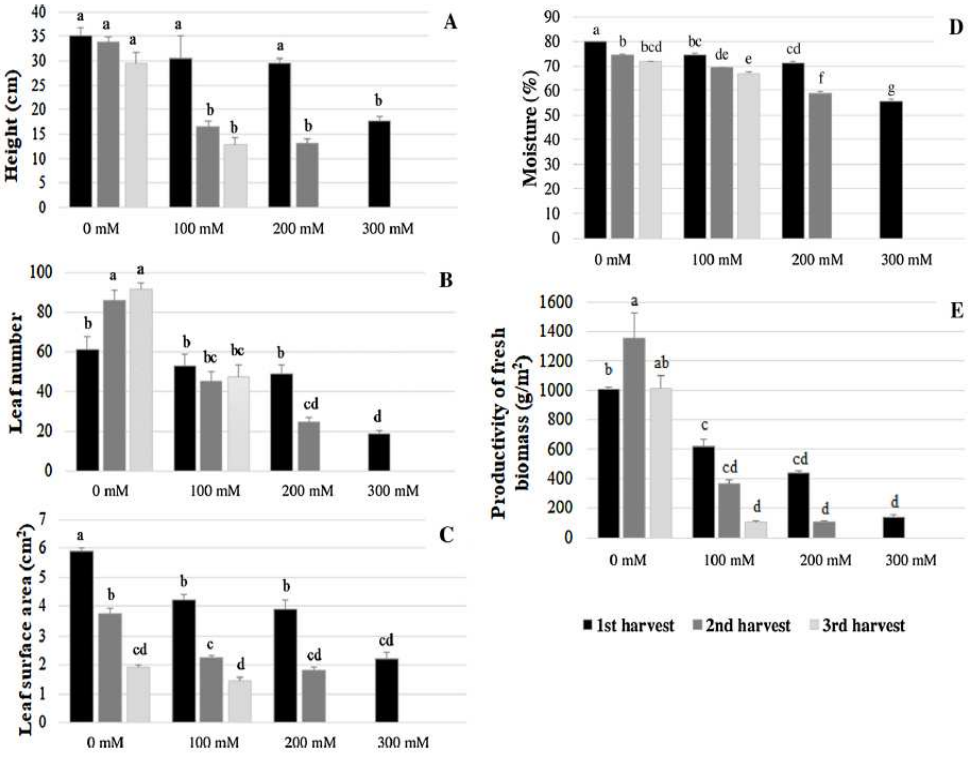

Fig. 1. Growth performance of sea knotgrass irrigated with freshwater (approximately 0 mM of NaCl), and artificial saltwater with different NaCl concentrations (100, 200 and 300 mM of NaCl), for 3 repeated harvests: A) height (cm), B) average leaf number, C) average leaf surface area (cm^2), D) moisture content (%), E) productivity of fresh plant biomass (g/m^2). Values represent the mean ± standard error of the mean (SEM) of 15 plants (n = 15). Columns with different letters are significantly different at $P < 0.05$ (Tukey HSD test).

saline conditions (Grigore et al., 2012). This can be explained by the fact that salinity is not the only limiting factor for halophytes, but also the availability of nutrients and water, indicating that the occurrence of these species in saline environments may be due only to competition avoidance with non-salt tolerant plants (Grigore et al., 2012). In turn, the salinity contributes to water deficit, reduced fresh weight, and plant growth, decreased photosynthesis, leaf injuries, nutritional deficiencies and physiological water stress (Koyro et al., 2008). Studies on other halophytes, such as *Ammophila arenaria* (L.) Link, *Elymus farctus* (Viv.) Runemark ex Melderis, and *Cakile maritima* Scop., that share the same growing habitat with sea knotgrass (beach and incipient dunes), have shown that increasing soil salinity negatively affects plant growth performance, causing reduced biomass, leaf number and plant height (Sykes and Wilson, 1989; Ksouri et al., 2007; van Puijenbroek et al., 2017). For instance, to avoid salt toxicity some halophytes shed the old leaves, which may explain the reduction of the leaf number observed in this work with increasing irrigation salinity of the sea knotgrass plants (Mishra and Tanna, 2017; van Puijenbroek et al., 2017). Similar to our results, the halophytes *Atriplex hortensis* L. and *C. maritima* also displayed reduced leaf area with increasing irrigation salinity (Ksouri et al., 2007; Sai Kachout et al., 2009). However, in contrast to what was observed in this work, the halophyte *A. arenaria* showed increased plant height with increasing soil salinity (van Puijenbroek et al., 2017).

In halophytes reduced research has been made on the effect of multiple harvest regimes on biomass production and properties. Gallagher (1985) studied the productivity of *Spartina patens* (Aiton) Muhl and a reduction of productivity was observed after repetitive harvests, which was similar to the results obtained in this study. In another study, Ventura and co-workers compared the growth parameters of multi harvesting of *Salicornia persica* Akhaniand and *Sarcocornia fruticosa* (L.) A.J. Scott, and disclosed that for *S. persica*, a reduced harvesting interval induced a decline on the yield, while for *S. fruticosa* the cutting regime did not affect the productivity (Ventura et al., 2011). Thus, to improve the sea knotgrass productivity when irrigated with saline water, longer harvesting intervals should be tested to allow plant recovery between harvests.

Despite the limited growth of sea knotgrass under saline conditions, plants survived in irrigation salinities up to 100 mM of NaCl, and resisted to the repeated harvests, suggesting that this species may be suitable for saline agriculture, using brackish waters or salinized soils. However, more detailed experiments are needed, as stated above, to select the best harvesting regime in saline conditions to increase productivity, as well to optimize other cultivation conditions, such as fertilization.

3.2. Functional properties of obtained biomass: in vitro antioxidant and anti-inflammatory activities

In a previous work, an ultrasound-assisted extraction technique with acetone, for 30 min, proved to be the best method to obtain a phenolic and flavonoid-enriched extract from the sea knotgrass with improved biological properties (Rodrigues et al., 2019). This method was therefore here used to prepare acetone extracts from produced biomass, which were further analysed for *in vitro* antioxidant properties through five complementary methods, namely radical scavenging activity of DPPH and ABTS, metal chelation of iron (ICA) and copper (CCA), and ferric reducing antioxidant power (FRAP). Results are shown in Fig. 2 and in Rodrigues et al. (data in brief, submitted, Table 1).

It was observed an increase in the RSA in the extracts made from biomass resulting from the 1^{s}t to 2nd and 3rd harvests for all salinity conditions, except for the 100 mM treatment on ABTS that decreased on

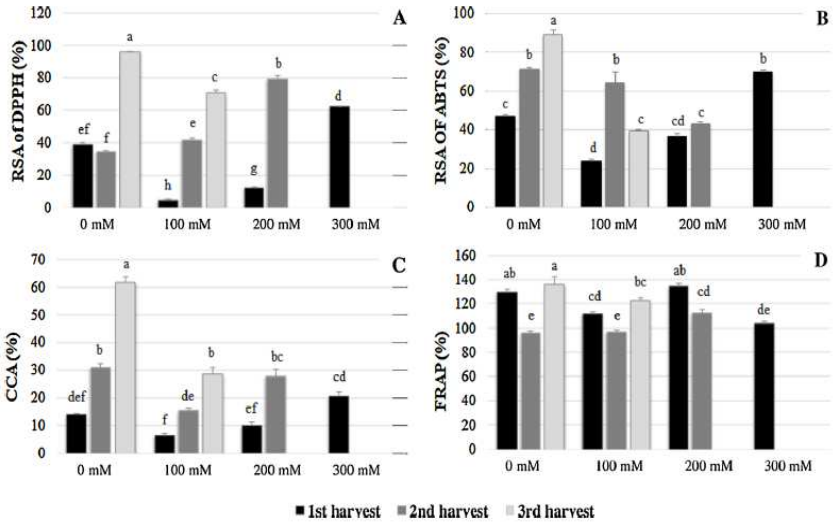

Fig. 2. *In vitro* antioxidant activity (%) of sea knotgrass irrigated with freshwater (approximately 0 mM of NaCl) and artificial saltwater with different NaCl concentrations (100, 200 and 300 mM), for 3 repeated harvests, at the concentration of 1 mg/mL. A) radical-scavenging activity (RSA) of DPPH$^{\bullet}$, B) RSA of ABTS$^{+\bullet}$, C) copper chelating activity (CCA), D) ferric reducing antioxidant power (FRAP). Values represent the mean ± standard error of the mean (SEM) of at least three experiments each performed in triplicate (n = 9). Columns with different letters are significantly different at $P < 0.05$ (Tukey HSD test).

Table 1
Total contents of phenolics (TPC) and flavonoids (TFC) of acetone extracts of *P. maritimum* aerial parts irrigated with freshwater and artificial saltwater with different NaCl concentrations, for 3 repeated harvests.

		Salinity irrigation[1]			
Group of phenolics	Harvest	0	100	200	300
TPC (mg GAE/g DW)	1st	107 ± 4^{b}	85.7 ± 2.9^{bcd}	96.4 ± 4.4^{bc}	107 ± 8^{b}
	2nd	55.5 ± 6.3^{cde}	16.0 ± 1.3^{e}	43.4 ± 4.9^{de}	–
	3rd	251 ± 21^{a}	64.0 ± 1.9^{bcd}	–	–
TFC (mg RE/g DW)	1st	17.2 ± 1.6^{cd}	15.8 ± 1.1^{bcd}	15.0 ± 1.7^{d}	16.5 ± 2.7^{cd}
	2nd	4.87 ± 0.61^{e}	21.3 ± 0.5^{bc}	26.1 ± 1.8^{b}	–
	3rd	28.5 ± 0.8^{a}	16.8 ± 1.3^{cd}	–	–

Values represent the mean ± standard error of the mean (SEM) of at least three experiments each performed in triplicate (n = 9). Statistical analysis was made separately for each assay (TPC and TFC) and the values followed by different letters are significantly different at $P < 0.05$ (Tukey HSD test). DW: dry weight; RT: room temperature; GAE: gallic acid equivalents; RE: rutin equivalents; TPC: mg GAE/g DW; TFC: mg RE/g DW.
-: samples that did not survive until the harvest.
[1] in mM of NaCl.

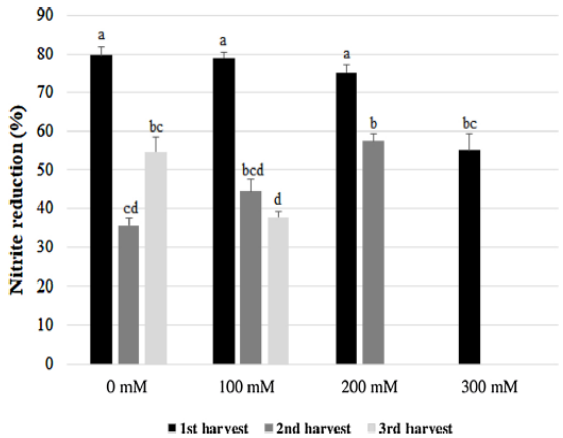

Fig. 3. *In vitro* anti-inflammatory activity (%) of sea knotgrass irrigated with freshwater (approximately 0 mM of NaCl) and saltwater with different NaCl concentrations (100, 200 and 300 mM), for 3 repeated harvests, at the concentration of 100 µg/mL. Results are expressed as a percentage (%) of nitric oxide decrease relative to a control with the vehicle (0.2% DMSO, v/v). Values represent the mean ± standard error of the mean (SEM) of least three experiments each performed in triplicate (n = 9). Columns with different letters are significantly different at $P < 0.05$ (Tukey HSD test).

the 3rd harvest. For the three assays, freshwater-irrigated plants at the 3rd harvest showed the highest RSA, with values of 96.2, 89.1 and 61.6% at 1 mg/mL, and IC_{50} values of 138, 279 and 560 µg/mL, for DPPH, ABTS, and CCA, respectively (Fig. 2A, B, C and Rodrigues et al., (data in brief, submitted, Table 1). For all conditions, FRAP reduced from the 1st to the 2nd harvests but increased again in the 3rd cut, and amid all, the 3rd harvest of plants irrigated with freshwater showed the highest FRAP at 1 mg/mL (136%; Fig. 2D), and the lowest IC_{50} value (54 µg/mL; Rodrigues et al. (data in brief, submitted, Table 1). None of the samples showed the capacity to chelate iron ions (data not shown). The different patterns of activity observed for the different methods, may be related to the different mechanisms of action of the bioactive compounds regarding the diverse reactive species, *i.e.*, the same molecule may exhibit a different behaviour against the various oxidative agents, and thus the compounds present dissimilar activities against the same oxidant (Dai and Mumper, 2010; Noguchi and Niki, 2000). This reinforces the need to use several methods for determining the antioxidant potential of a sample since oxidative stress may be related to different reactive species (Badarinath et al., 2010; Niki, 2010). Moreover, and since raw extracts are complex mixtures of different compounds, often showing synergistic and antagonistic interactions, is difficult to predict exactly which compounds are responsible for the detected activity. Regarding the increased FRAP activity observed from 2nd to 3rd harvest, one possible reason, according to the data obtained in this work, may be the increase in 5 °C on the minimum temperature in

Table 2
UHRMS tentative identification of metabolites present in acetone extracts of *P. maritimum* aerial parts irrigated with freshwater (0 mM) and artificial saltwater with different NaCl concentrations (100, 200 and 300 mM), for 3 repeated harvests.

Peak no.[a]	Compound[b]	t_R[c]	m/z[M-H]$^-$	Formula	MS^2 main ion	Fragments	1st harvest				2nd harvest			3rd harvest	
							0[d]	100	200	300	0	100	200	0	100
1	6-O-Galloyl-glucose	0.9	331.0673	$C_{13}H_{16}O_{10}$	169	271,211,151									
2	Citric acid	1.0	191.0198	$C_6H_8O_7$											
3	Gallic acid	1.1	169.0137	$C_7H_6O_5$	125										
4	5-Acetyl-2,3-dihydroxyphenyl beta-D-glucopyranoside	1.3	329.0880	$C_{14}H_{18}O_9$	167	152									
5	**Hydroxy-gallic acid**	**1.5**	**187.0608**	**$C_8H_{12}O_5$**	**169**	**170,152,125**	+ +	+ + +			+ + +				+ +
6	NI	1.8	182.0215	$C_8H_6O_5$	167	123,152									
7	Coumaroyl-O-glucose	2.0	325.0925	$C_{15}H_{18}O_8$	163	119									
8	Dihydroferulic acid 4-O-glucuronide	2.9	371.0990	$C_{16}H_{20}O_{10}$	163	195									
9	D-threo-Hexitol	3.0	293.1247	$C_{12}H_{22}O_8$	131										
10	**Methylgallic acid-O-sulphate**	**2.5-3.5**	**262.9863**	**$C_8H_8O_8S$**	**183**						+ + +	+ + +		+ + +	+ + +
11	Hydroxycinnamic acid-glucoside	3.6	325.0932	$C_{15}H_{18}O_8$	163	119									
12	1-Caffeoyl-beta-D-glucose	4.0	341.0870	$C_{15}H_{18}O_9$	179	135									
13	NI	4.2	431.1920	$C_{20}H_{32}O_{10}$	223	179,164									
14	**Caffeic acid 3-sulfate**	**4.5**	**258.9910**	**$C_9H_8O_7S$**	**179**	**135**	+ +				+ +			+ +	
15	1-O-Feruloylglucose	5.5	355.1030	$C_{16}H_{20}O_9$	175	193,161									
16	**1-O-Sinapoyl-beta-D-glucose**	**6.5**	**385.1142**	**$C_{17}H_{22}O_{10}$**	**205**	**190,164**	+								
17	Coumaroylquinic acid	7.5	337.0933	$C_{16}H_{18}O_8$	191	173,163									
18	NI	7.7	551.1768	$C_{26}H_{32}O_{13}$	341	193,165									
19	NI	8.0	433.2082	$C_{20}H_{34}O_{10}$	387	161									
20	**Coumaroylquinic acid**	**8.4**	**337.0933**	**$C_{16}H_{18}O_8$**	**191**	**173,163**						+ + +			
21	**Taxifolin-3-glucopyranoside**	**9.5**	**465.1037**	**$C_{21}H_{22}O_{12}$**	**303**	**313,151,419,404,373**				+ +			+ +		
22	a deoxyglucoside of hydroxylated furanocoumarin derivative	9.7	379.1022	$C_{18}H_{20}O_9$	319	233,173,191,145									
23	NI	9.8	551.2714	$C_{25}H_{44}O_{13}$	505	327,251,191,149									
24	Myricetin 3-O-rhamnoside	10.1	463.0889	$C_{21}H_{20}O_{12}$	316	300									
25	**Quercetin-3-O-galactoside**	**10.5**	**463.0885**	**$C_{21}H_{20}O_{12}$**	**300**		+ + +	+ + +	+ + +		+ + +	+ + +		+ +	+ + +
26	Quercetin-3-O-glucoside	10.6	463.0891	$C_{21}H_{20}O_{12}$	300										
27	**Quercitrin**	**11.0**	**447.0939**	**$C_{21}H_{20}O_{11}$**	**301**	**285**	+ + +	+ + +	+ +	+ +	+	+ +	+ + +	+	+ + +
28	Myricetin-3-O-β-D-(6"-O-galloyl)-galactopyranoside	11.7	631.0974	$C_{28}H_{24}O_{17}$	345	551,223,183									
29	Viscumneoside VI	12.1	505.1346	$C_{24}H_{26}O_{12}$	367	316,205,353,137									
30	Isolimocitrol 3-glucoside	12.2	537.1247	$C_{24}H_{26}O_{14}$	169										
31	NI	12.2	431.1914	$C_{20}H_{32}O_{10}$	251	207									
32	NI	12.3	312.1227		297	190,178,148,135									
33	NI	12.4	775.2812	$C_{38}H_{48}O_{17}$	565	417,387,165,195									
34	NI	12.5	745.2706	$C_{37}H_{46}O_{16}$	535	387,165,195									
35	NI	12.6	531.1504	$C_{26}H_{28}O_{12}$	367	300,205,163									
36	NI	13.0	583.3332	$C_{27}H_{52}O_{13}$	375										
37	NI	13.2	671.1621		313										
38	**Quercetin**	**13.3**	**301.1651**	**$C_{16}H_{14}O_6$**				+	+		+	+ +	+	+	+ +
39	NI	13.4	531.1504	$C_{26}H_{28}O_{12}$	353	361,163,191,205,177									
40	NI	13.6	581.3162	$C_{27}H_{50}O_{13}$	373										
41	NI	13.7	375.2028	$C_{18}H_{32}O_8$	195										
42	**NI**	**13.9**	**447.2296**		**171**	**149,191,251,221**							+ +		
43	NI	14.8	507.2078	$C_{22}H_{36}O_{13}$	293	233,149,167,125									
44	NI	15.5	375.1659	$C_{17}H_{28}O_9$	173										
45	**9,10-Dihydroxy-8-oxo-12-octadecenoic acid**	**16.5**	**327.2168**	**$C_{18}H_{32}O_5$**	**211**	**171,229,309**	+	+ +	+ + +				+ + +		
46	**9,10,13-trihydroxyoctadecanoic acid**	**17.5**	**331.2476**	**$C_{18}H_{36}O_5$**	**313**		+ +	+ + +	+ + +				+ + +	+ + +	
47	**(S)-10,16-Dihydroxyhexadecanoic acid**	**19.9**	**287.2213**	**$C_{16}H_{32}O_4$**			+ +	+ + +			+ + +	+ + +		+ + +	

Marker compounds (in bold) were identified for distinguishing groups of experiments and occurrence (+, + + or + + + for low, medium or high abundance, respectively).
[a] Corresponding peak number in the chromatogram on Fig. 4.
[b] Identified by comparison of the retention parameters with the standard controls and peak purity with the UV–vis spectral reference data. nd: not detected.
[c] Retention times (minutes).
[d] in mM of NaCl.

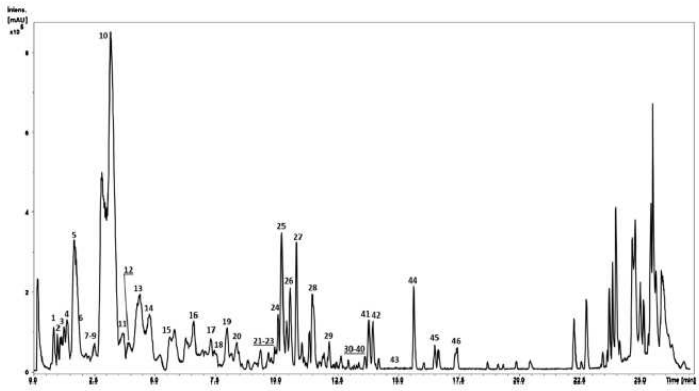

Fig. 4. Base peak chromatogram of the acetone extract from biomass obtained in the 1st harvest of plants irrigated with freshwater, showing the most important metabolites for their identification (detailed in Table 2).

the greenhouse that occurred on the 4 weeks that preceded the 3rd harvest. In fact it is known that the increase in temperature can influence the production of secondary metabolites, such as phenolics and flavonoids, and therefore the related biological activities (Chua et al., 2015; Shohael et al., 2006; Wang and Zheng, 2001).

Ksouri et al. (2007) reported that the RSA of DPPH of *C. maritima* methanol extracts, derived from seeds collected at Jerba, did not change by increasing salinity in the culture medium, but plants derived from seeds from Tabarka had decreased activity with increasing NaCl concentrations. In turn, Mansour et al. (2018) reported that the antioxidant activity of 80% methanol extracts from *C. maritima*, in the vegetative stage, improved with increasing salinity of the culture medium; when plants were at the flowering stage the activity decreased from control to 100 mM of NaCl, but increased again with the higher salt concentrations, similar to the results obtained in this work with sea knotgrass.

The *in vitro* anti-inflammatory capacity of the extracts was determined by their effect on NO reduction on LPS-stimulated RAW 264.7 macrophages, and the results are shown in Fig. 3 and Rodrigues et al., (data in brief, submitted, Table 2). The NO reduction was similar between extracts obtained from biomass from all the salinity treatments, for the first harvest ($P < 0.05$), with the highest anti-inflammatory activity values varying from 75.3 and 79.7% at 100 µg/mL (Fig. 3), and IC_{50} values between 51.4 and 53.7 µg/mL (Rodrigues et al. (data in brief, submitted, Table 2). Furthermore, the anti-inflammatory activity decreased from the 1st to the 2nd and 3rd harvests, except in freshwater-irrigated plants, where an increase was observed from the 2nd to the 3rd harvest (Fig. 3). This pattern is similar to that observed in the FRAP assay and could be related to the same issue, the increase in the minimum temperature that occurred 4 weeks before the 3rd harvest. As far as we could search, this is the first work reporting the possible relationship between the culture conditions and anti-inflammatory properties of halophyte plants.

3.3. Chemical composition

The chemical composition of sea knotgrass acetone extracts was analysed by spectrophotometric methods, namely total phenolics (TPC) and flavonoids (TFC) contents (Table 1), and ultra-high-resolution mass spectrometry (UHRMS) analysis (Table 2, Fig. 4 and Fig. S2).

Regarding TPC, a reduction from the 1st to the 2nd harvests was observed for all treatments, and for plants irrigated with freshwater and 100 mM salinity treatments, it was followed by an increase on the 3rd harvest. Amongst all irrigations conditions, the 3rd harvest of plants irrigated with freshwater showed the highest TPC (251 mg GAE/g DW), despite, the 1st harvest for the salinity treatments also had considerable levels of phenolics which increased with increasing salinity (100 mM: 85.7 mg GAE/g DW; 200 mM: 96.4 mg GAE/g DW; and 300 mM: 107 mg GAE/g DW; Table 1). For TFC, although the similar decreasing tendency from the 1st to the 2nd harvests in the control was observed, the total flavonoids increased at the 2nd harvest in the salinity treatments. Once again, the biomass obtained in the 3rd cut of plants irrigated with freshwater had the uppermost TFC (28.5 mg RE/g DW), followed by the 2nd harvest of 200 mM (26.1 mg RE/g DW) and 100 mM (21.3 mg RE/g DW). The synthesis of phenolics and flavonoids is induced by biotic and/or abiotic stresses (*e.g.* salinity, drought, ultraviolet (UV)-radiation and/or herbivory) once they are known to be produced as plant defence mechanisms, thus their levels rise when plants are subjected to environmental stresses, such as increasing salinity and harvesting (mimics herbivory) (Ksouri et al., 2007).

There is a reduced number of studies related to the influence of the saline irrigation conditions on the biological/chemical properties of produced biomass. Ksouri and co-workers tested the saline cultivation of the halophyte *C. maritima* under two NaCl concentrations (100 and 400 mM) and its effect on the total phenolics content and antioxidant activity (Ksouri et al., 2007). Regarding TPC, this study presented different results according to the origin of the seed (site of the collection): plants obtained from seeds collected from one site (Tabarka) exhibited a decrease of TPC with increased NaCl concentrations in the culture medium, while the opposite was observed with plants from seeds collected from Jerba. This latter showed similar results to that observed in the present study by the sea knotgrass, and also to those reported by Mansour et al. (2018) that compared *C. maritima* plants at two developmental stages (vegetative and flowering) submitted to 100 mM and 400 mM salt concentrations in the culture medium, and which reported, for both stages, augmented TPC and TFC with increasing NaCl concentrations.

The detailed profiling using LC–MS detected several marker compounds that allow to discriminate variance between treatments and harvest: hydroxy-gallic acid (**5**), methylgallic acid-O-sulphate (**10**), caffeic acid 3-sulfate (**14**), 1-O-sinapoyl-beta-D-glucose (**16**), coumaroylquinic acid (**20**), taxifolin-3-glucopyranoside (**21**), quercetin-3-O-galactoside (**25**), quercitrin (**27**), a trixydroxyoctadienoic acid (**45**), 9,10,13-trihydroxyoctadecanoic acid (**46**) and (S)-10,16-dihydroxyhexadecanoic acid (**47**) (Table 2 and Fig. 4). Among these, the major peaks corresponded to flavonols, with a predominance of myricetin (**24** and **28**) and quercetin glycosides (**25-27**). These molecules were previously described as main compounds in this species collected from the wild, and diverse biological activities are attributed to their presence, as for example antioxidant, anti-inflammatory, antidiabetic and anti-melanogenic (Rodrigues et al., 2017, 2018, 2019). Thus, their occurrence in the sea knotgrass cultivated plants may clarify the high

antioxidant and anti-inflammatory capacity of these samples.

Principal component (PCA) and partial least squares-discriminant (PLS-DA) analyses clearly indicate a separation amongst the different treatments for the same harvest (Rodrigues et al. [data in brief, submitted, Figs. 1–3]). Hyperoside (quercetin-3-O-galactoside, **25**) was a major flavonoid in most of the samples and it had the highest (of all detected flavonoids) score in differentiating between treatments within the 1st and 2nd harvest, being most relatively abundant at 100 mM (Rodrigues et al. [data in brief, submitted, Fig. 1]). In addition, a couple of late-eluting lipophilic compounds (**45-47**) were associated with differences between treatments, including free aliphatic fatty acids, which shown to increase the content with the increasing salinity. This effect agrees with a previous report showing that one of the main salinity adaptations of *P. maritimum* is the increase of fatty acid saturation since it reduces membrane permeability and conferring higher salinity resistance of halophytic plants (Kosakivska et al., 2017). It is, however, unlikely that these constituents would determine the antioxidant and anti-inflammatory activity of these samples once they are described with the opposite effect, acting as pro-inflammatory molecules (Harvey et al., 2010).

Moreover, comparing the presence of the marker compounds amongst treatments, caffeic acid 3-sulfate (**14**) permits to distinguish between harvests, in the same irrigation treatment, since it was detected at a high amount on 1st harvest but declined on 2nd and 3rd harvests (Table 2). In turn, the tentatively assigned methylgallic acid-O-sulphate (**10**; based on accurate mass and fragmentation - *m/z* 262.9863) permits to differentiate from 1st to 2nd and 3rd harvests for the salinity treatments (Table 2). However, the identity of this peak, not detected before in this species, should be confirmed in the future by its isolation and full structure elucidation. Though, some sulfated metabolites are described to exhibit plant defence functions against pathogens and herbivores, which can explain the upsurge of this compounds after the 1st harvest as a protective mechanism against the harvesting, which is a way of simulated herbivory (Sánchez-Sánchez and Morquecho-Contreras, 2017).

However, it should be borne in mind that the above-listed compounds have been identified based on literature data and mass spectra analysis. The literature on *P. maritimum* is insufficient, so reports on several related knotweed species were used that could account for potential variation in the polyphenol profile (Mahmoudi et al., 2018, 2019). For ultimate identification, the most important compounds should be confirmed by spectroscopic techniques following their isolation.

We also observed differences in the extract composition from our previous study using wild *P. maritimum* plants, mainly seen in a marked predominance of myricitrin and catechin in the wild material, compared with the more variable profile without the significant presence of catechin and with a larger proportion of quercetin glycosides in most of the treatments. Besides the adaptive mechanism putatively causing accumulation of sulphated derivatives, the apparent dysregulation of the flavonol biosynthesis would indicate a complex response that needs further investigation including monitoring of biosynthetic genes expression and redox status of plants under stress. The shift towards less hydroxylated structures may result from altered activity or/and expression of respective oxidases/dehydrogenases under stress (Fini et al., 2011; Chapman et al., 2019). An increased decay of highly hydroxylated myricetin glycosides due to more oxidant milieu in the cells is another hypothetical explanation for the prevalence of more oxidatively stable quercetin. On the other hand, high content of myricetin – the superior singlet oxygen scavenger, in wild plants exposed to strong sunlight and heat might be an adaptive trait which has not been maintained in plants cultivated in the greenhouse (Csepregi and Hideg, 2018). Consequently, the complex environmental factors must be considered in optimizing agronomy of this halophyte to obtain desirable phytochemical profile and thereby determined bioactivity.

4. Conclusions

This is the first work reporting the greenhouse production of the halophyte sea knotgrass irrigated with water with different salinities (0, 100, 200, 300 and 600 mM of NaCl), submitted to successive harvests, and the relationship between those conditions and the growth performance and the *in vitro* antioxidant, anti-inflammatory and chemical profiles of obtained biomass. The increased salinity of the irrigation water and the multiple harvesting regimes reduced the plant growth and yields, and plants irrigated with freshwater had the highest productivity. The acetone extracts made with biomass collected from the different treatments had *in vitro* antioxidant and anti-inflammatory activities, as well as an interesting chemical profile, which were dependent on the irrigation salinity and harvest regime. The highest antioxidant activities were generally shown by freshwater-irrigated plants at the 3rd harvest, but regarding anti-inflammatory activity either the plants of the 1st harvest of freshwater-irrigated plants, as those treated with 100 and 200 mM of NaCl showed a high reduction on nitrites production by LPS-stimulated RAW 264.7 macrophages. The main compounds detected were flavonols (myricetin and quercetin glycosides).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111510.

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SUPPLEMENTARY MATERIAL

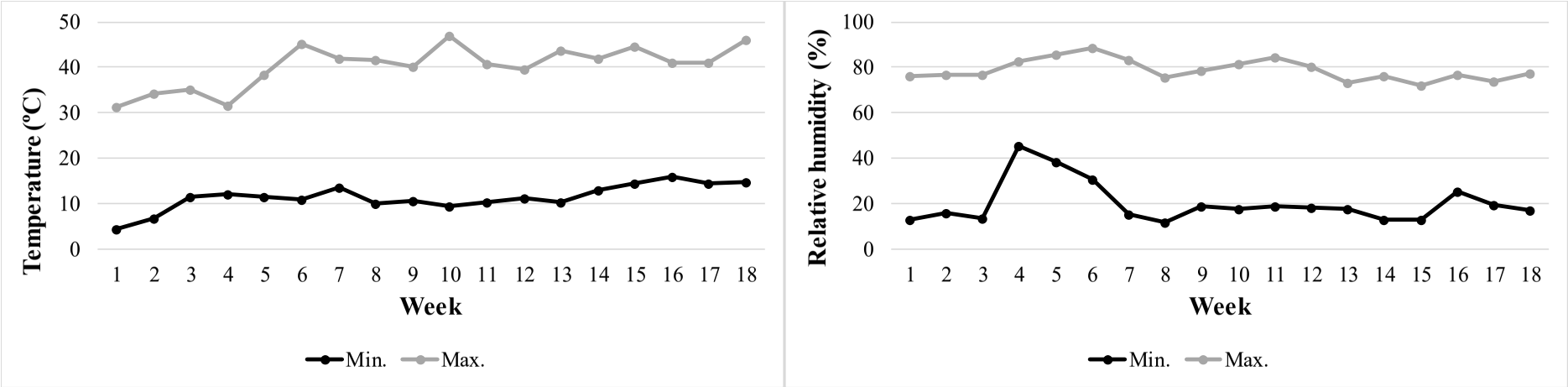
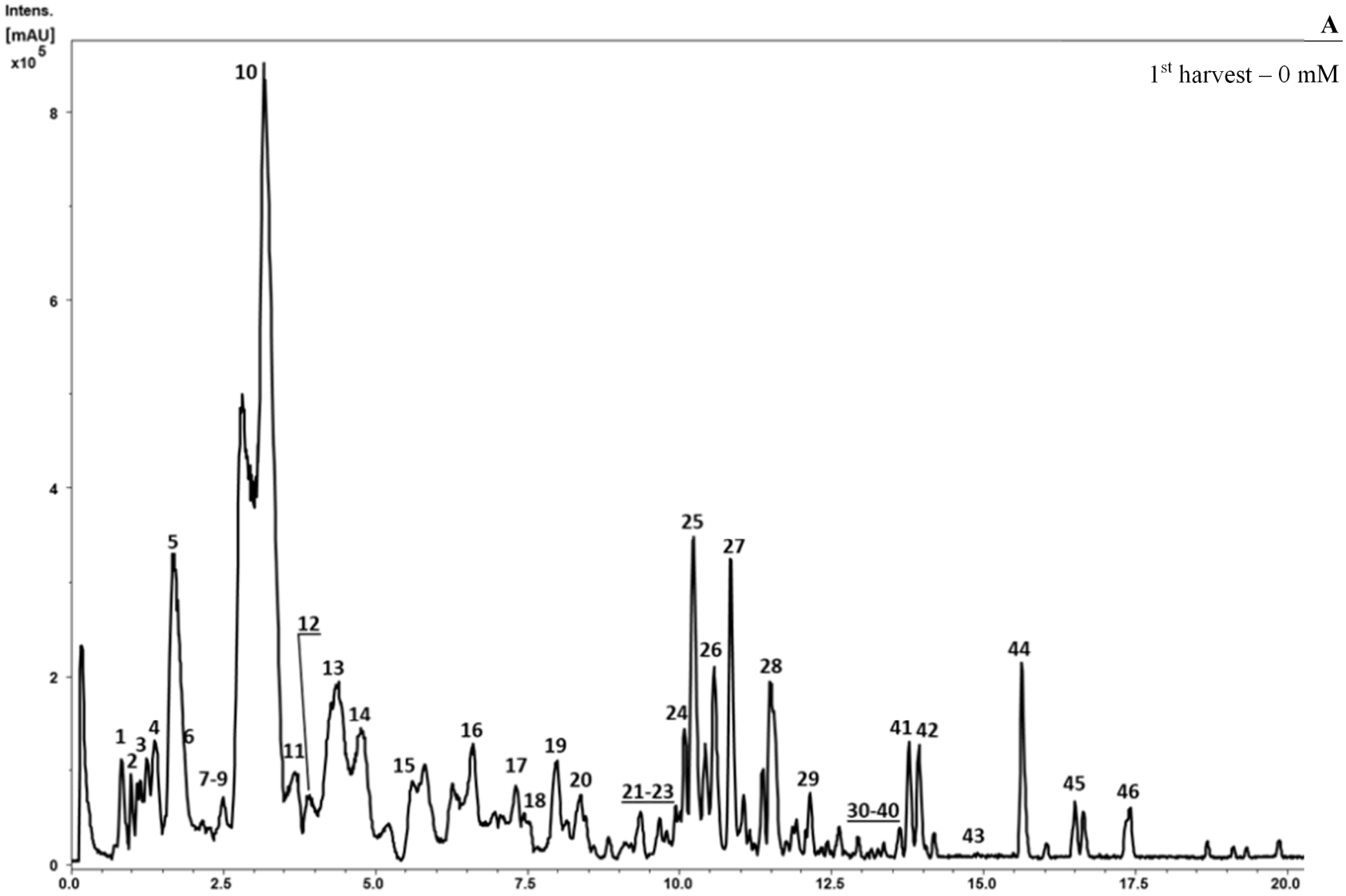


Fig. S1. Minimum and maximal temperature (**A**) and relative humidity (**B**) of the greenhouse during the 18 weeks of sea knotgrass cultivation.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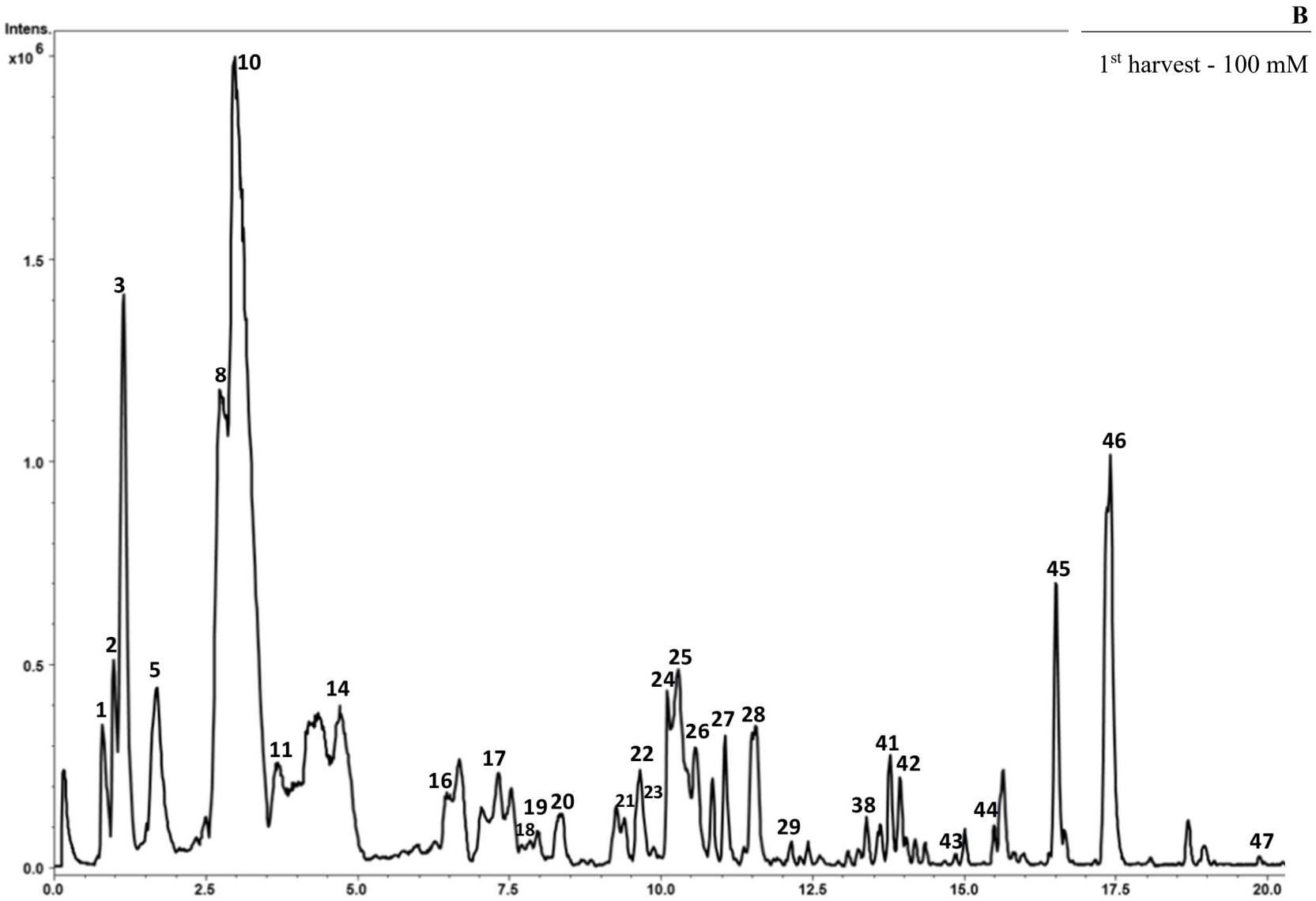
B
1st harvest - 100 mM
Intens.
x10 6
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
1
2
3
5
8
10
11
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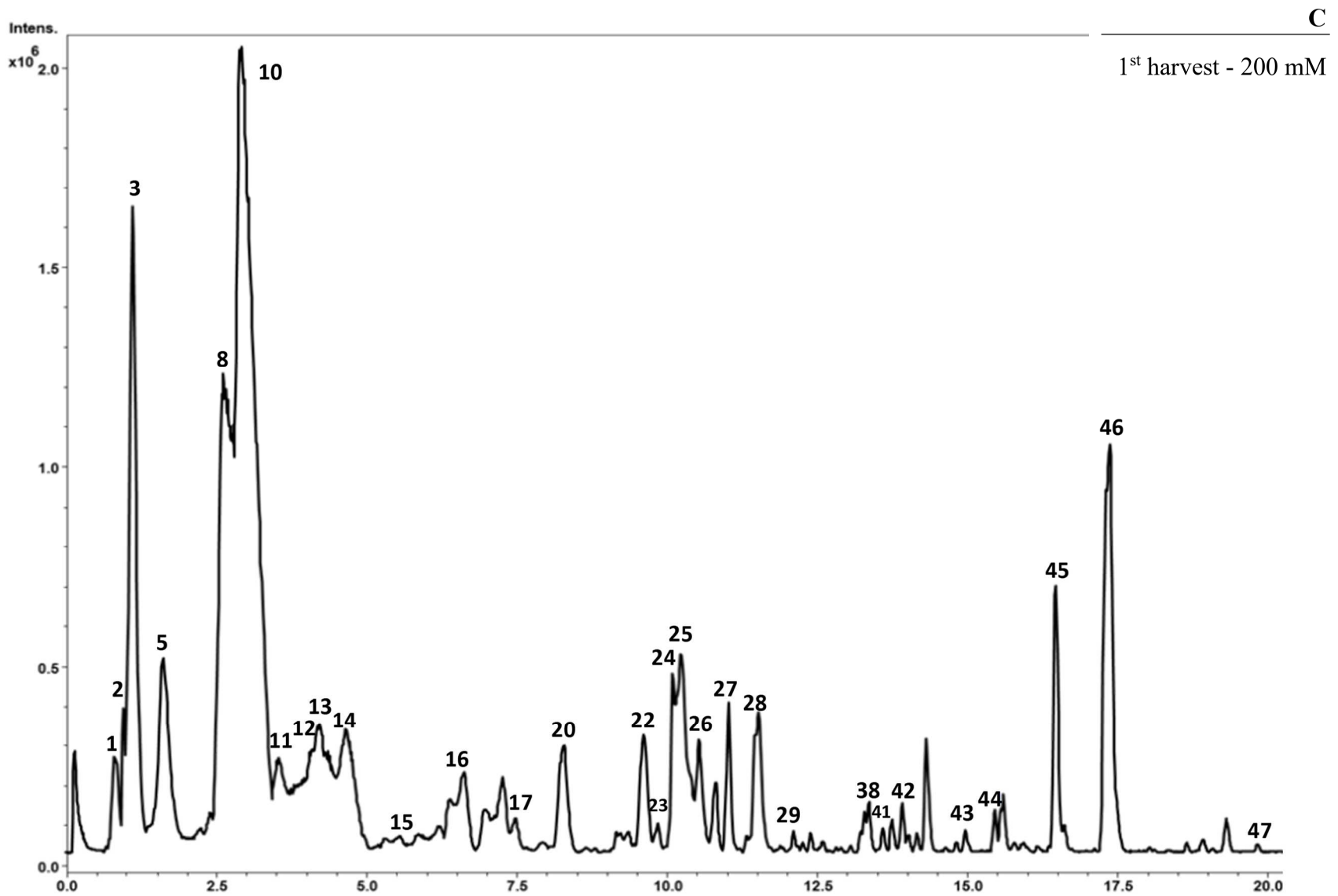
C
1st harvest - 200 mM
Intens.
x10^6
2.0
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
1
2
3
5
8
10
11
12
13
14
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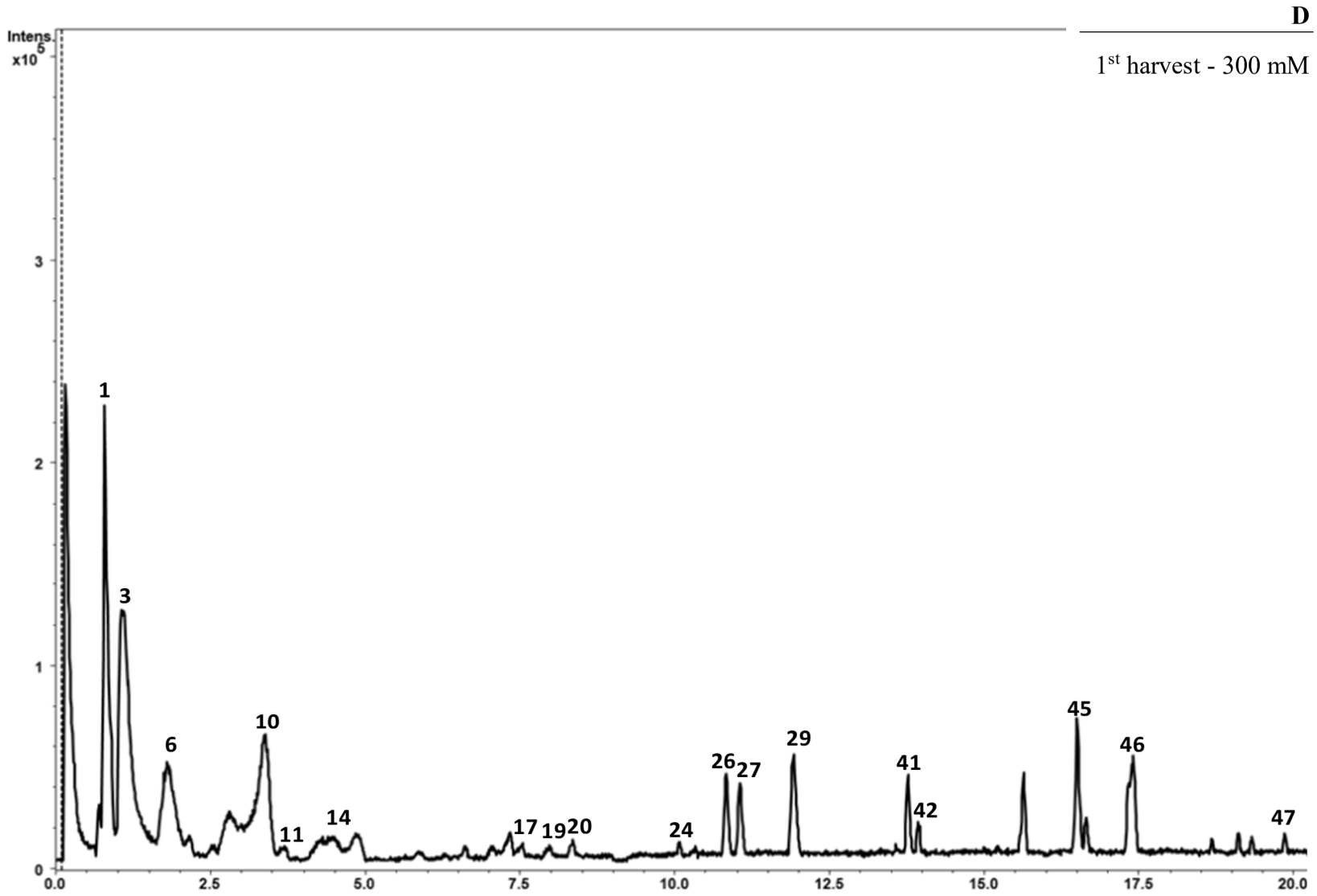
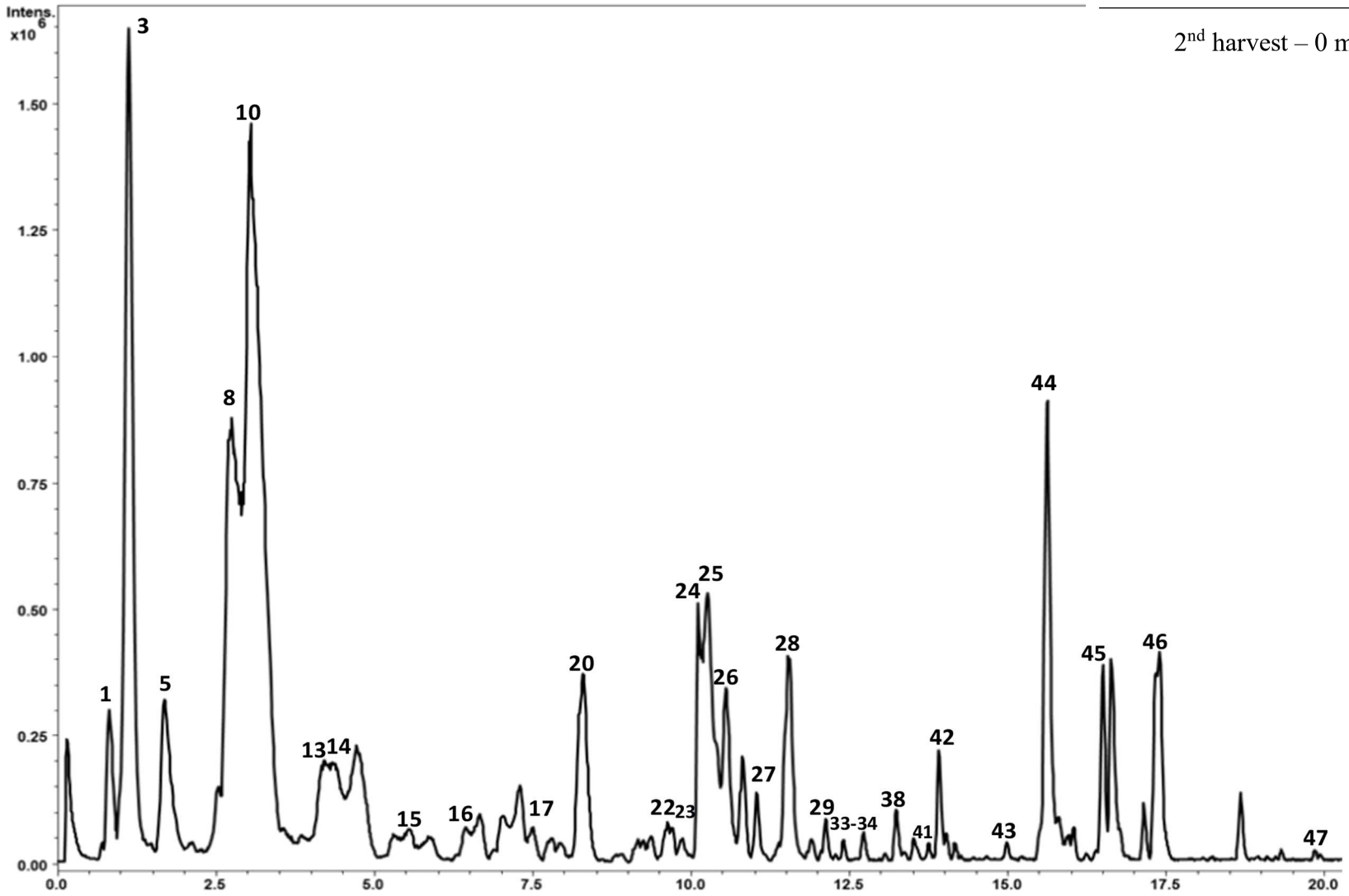
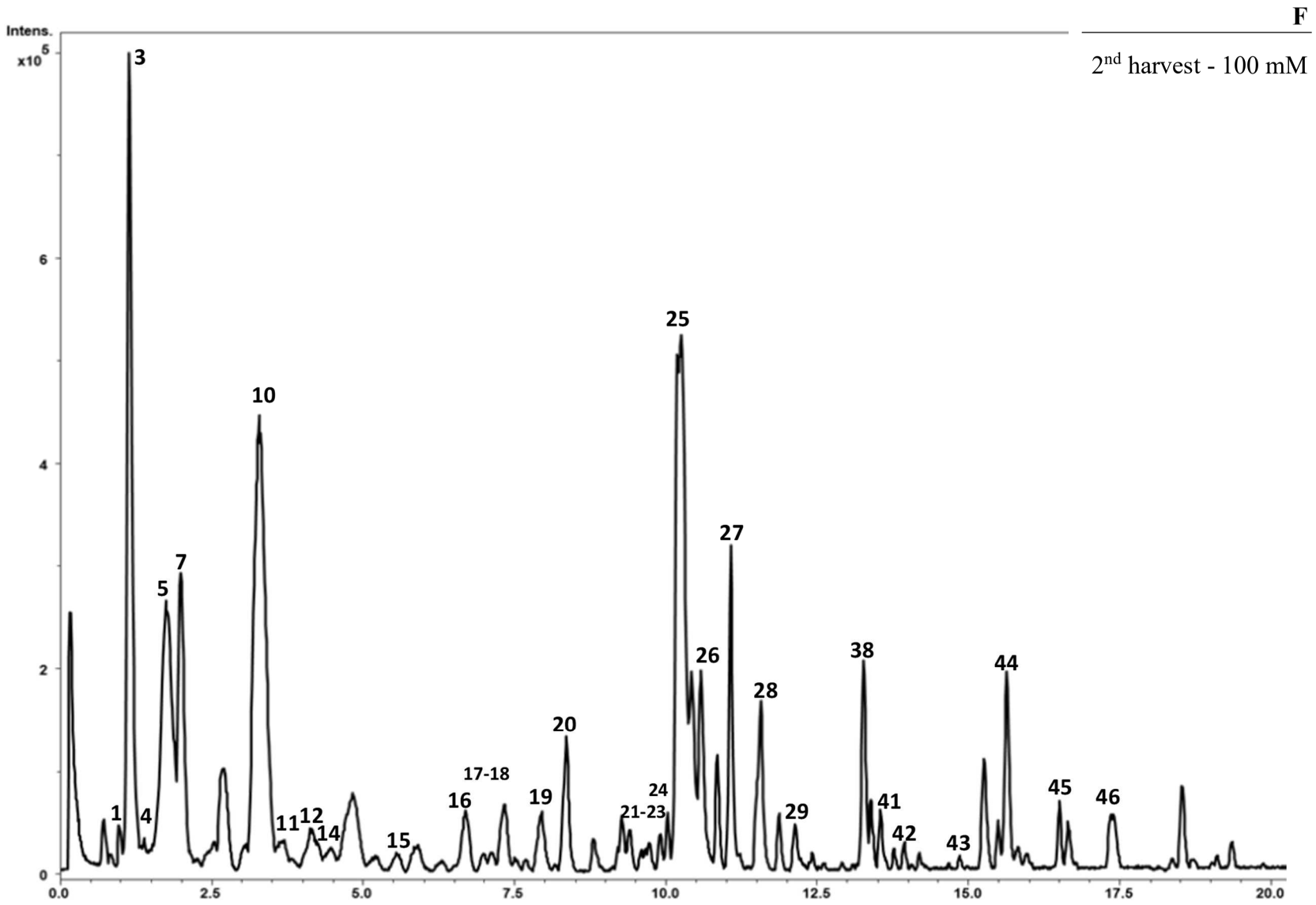
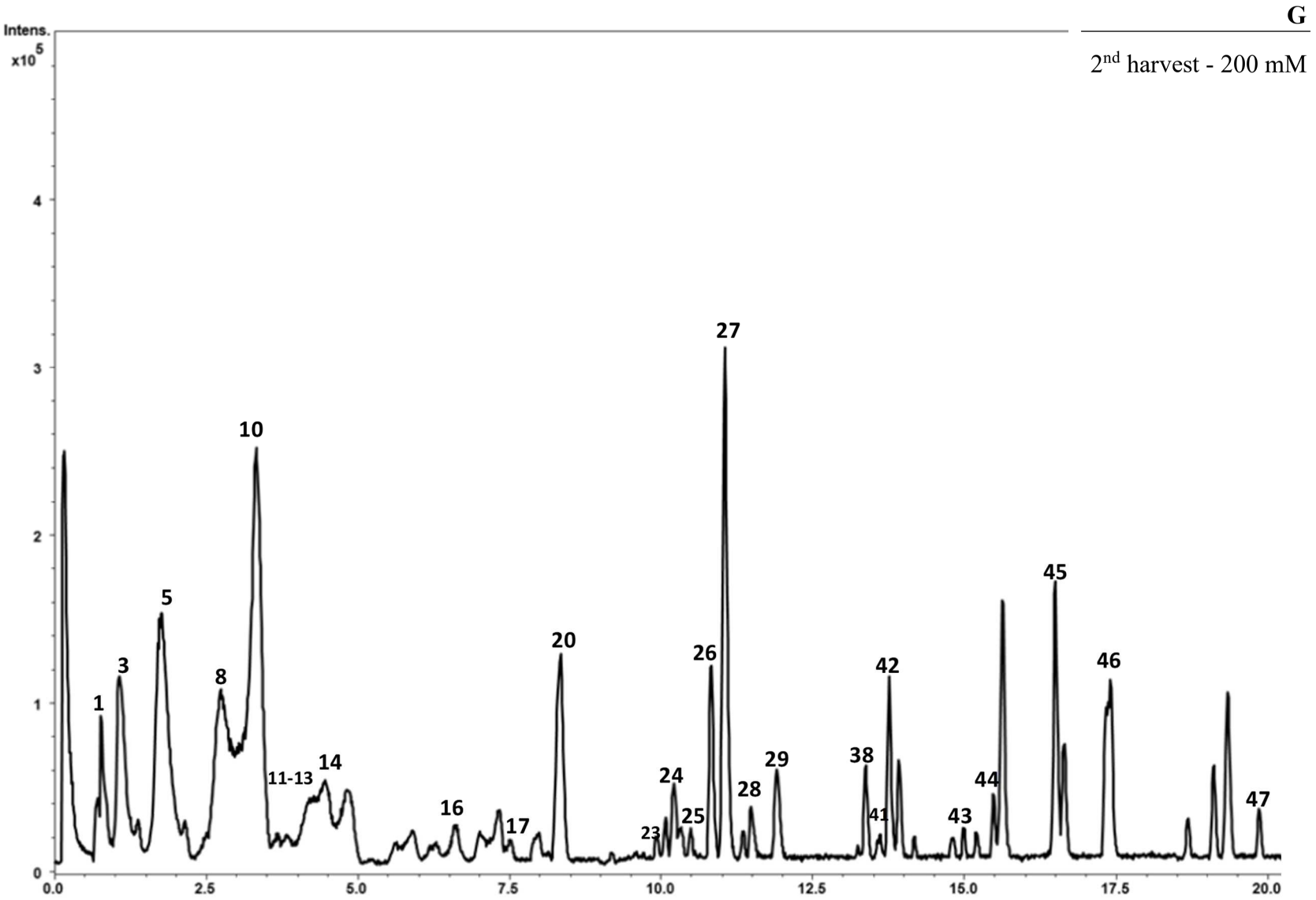

D
1st harvest - 300 mM
Intens.
x10^5
3
2
1
0
1
3
6
10
11
14
17
19
20
24
26
27
29
41
42
45
46
47
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0

E

2nd harvest – 0 mM

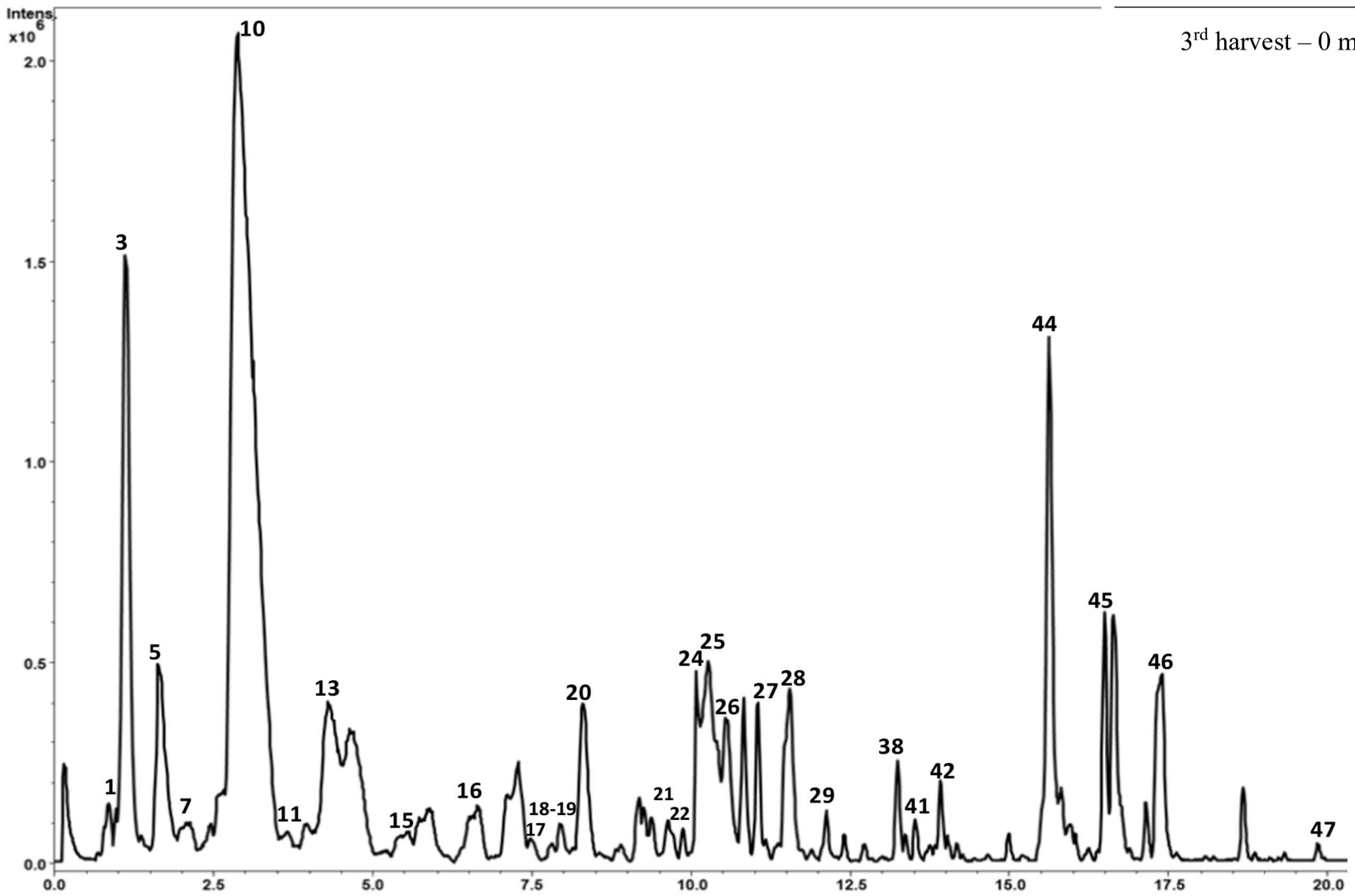



F
2nd harvest - 100 mM
Intens.
x10^5
0
2
4
6
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
1
3
4
5
7
10
11
12
14
15
16
17-18
19
20
21-23
24
25
26
27
28
29
38
41
42
43
44
45
46


G
2nd harvest - 200 mM
Intens.
x10^5
4
3
2
1
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
1
3
5
8
10
11-13
14
16
17
20
23
24
25
26
27
28
29
38
41
42
43
44
45
46
47

H

3rd harvest – 0 mM



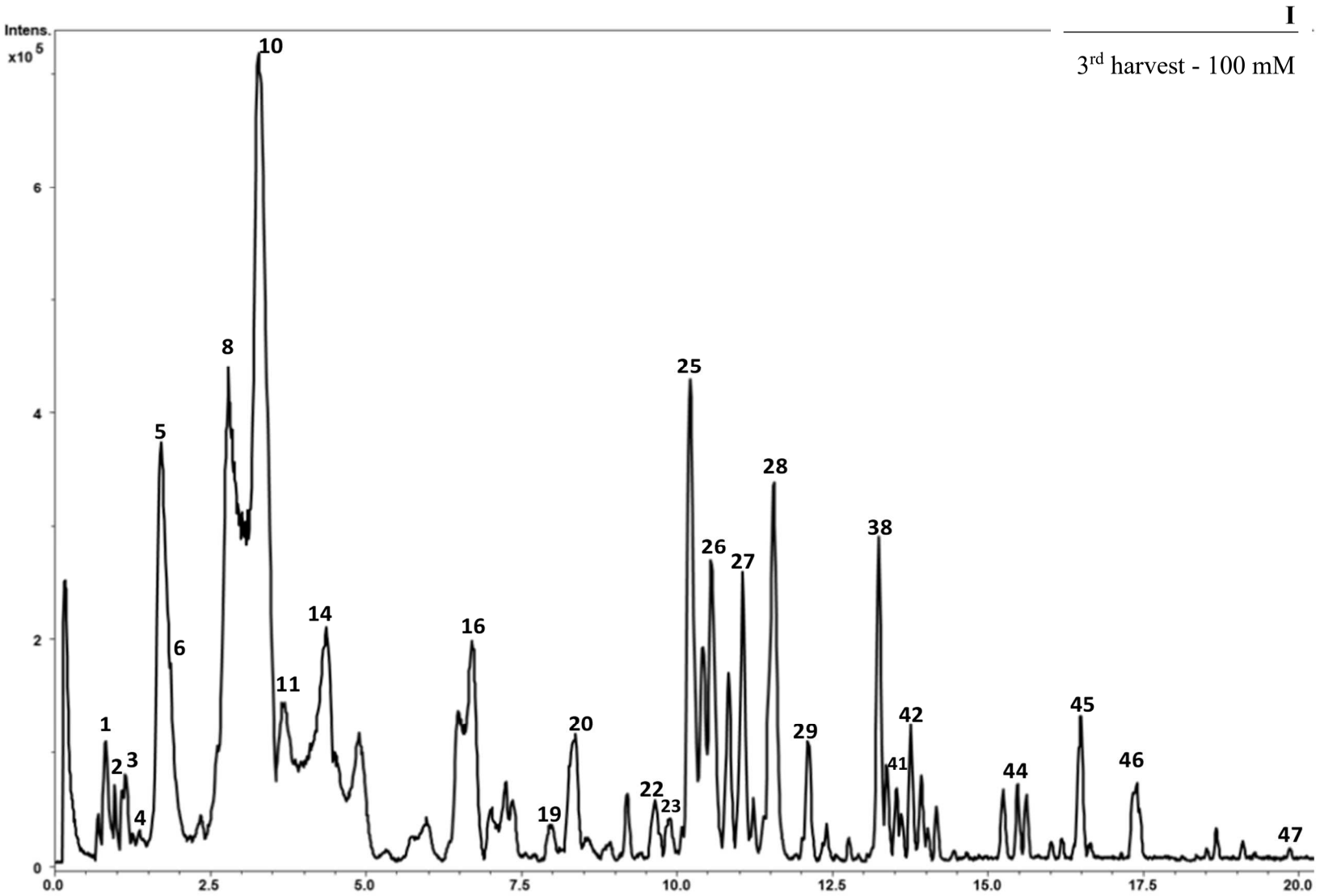


Fig. S2. Chromatograms of acetone extracts from of sea knotgrass irrigated with freshwater (0 mM of NaCl) and saltwater with different NaCl concentrations (100, 200 and 300 mM), for 3 repeated harvests. Peak number correspond to compounds listed in Table 2 of the main text.

Data in Brief xxx (xxxx) xxx-xxx

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Data in Brief

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Data Article

Dataset on functional and chemical properties of the medicinal halophyte *Polygonum maritimum* L. under greenhouse cultivation

Maria João Rodrigues [a], Ivo Monteiro [b], Chloé Placines [a], Viana Castañeda-Loaiza [a], Sylwester Ślusarczyk [c], Adam Matkowski [c], Catarina Pereira [a], Pedro Pousão-Ferreira [b], Luísa Custódio [a, *]

[a] *Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139, Faro, Portugal*
[b] *IPMA, Aquaculture Research Station, Olhão, Portugal*
[c] *Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Wroclaw, Poland*

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ABSTRACT

This data article includes data and analyses on the effect of different agronomic techniques on the production of *Polygonum maritimum* L. (sea knotgrass), namely different salinity irrigation treatments (0, 100, 200, 300 and 600 mM of NaCl) and a multi-harvest regime, and their relation with the chemical profile (ultra-high-resolution mass spectrometry - UHRMS), *in vitro* antioxidant [radical-scavenging activity (RSA) of DPPH and ABTS, copper chelating activity and ferric reducing antioxidant power] and anti-inflammatory (nitric oxide reduction on lipopolysaccharide-stimulated macrophages) activities. For further interpretation of the data presented in this work, please see the related research article "The irrigation salinity and harvesting affect the growth, chemical profile and biological activities of *Polygonum maritimum* L." (Rodrigues et al., 2019).

Specifications Table

Subject area	Agronomy, biology, chemistry
More specific subject area	Biological and chemical profiling of *Polygonum maritimum* cultivated under saline irrigation
Type of data	Table, graph, figure
How data was acquired	Photospectrometer (Biotek synergy 4), liquid chromatography (LC) – electrospray ionization (ESI)-QTOF-MS) (Thermo Dionex Ultimate 3000 RS).
Data format	Raw and analyzed
Experimental factors	12-week plants were subjected to different salinity irrigation treatments (freshwater, 100, 200, 300 and 600 mM of NaCl), followed by a multi harvesting regime with a 6-week interval.
Experimental features	Plants from different conditions were freeze-dried and resultant biomass extracted with acetone (1:40, w/v) and analyzed for their *in vitro* antioxidant and anti-inflammatory properties and chemical profile by LC-UHRMS.

* Corresponding author.
Email address: lcustodio@ualg.pt (L. Custódio)

https://doi.org/10.1016/j.dib.2019.104357
0926-6690/

Data source location	Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal.
Data accessibility	Data provided within this article.
Related research article	M.J. Rodrigues, I. Monteiro, C. Placines, V. Castañeda-Loaiza, S. Ślusarczyk, A. Matkowski, C. Pereira, P. Pousão-Ferreira, L. Custódio, The irrigation salinity and harvesting affect the growth, chemical profile and biological activities of *Polygonum maritimum* L., Ind. Crop. Prod. 139 (2019) 111 510. https://doi.org/10.1016/j.indcrop.2019.111510 [1].

Value of the data

- The first dataset on the effect of agronomic techniques (irrigation salinity and harvesting) on the chemical profile and *in vitro* antioxidant and anti-inflammatory properties of the medicinal halophyte *P. maritimum* (sea knotgrass)
- This dataset provides relevant information to other researchers for understanding the influence of cultivation conditions, including saline irrigation and multi-harvest regime, on halophyte plants functional properties
- Data could be relevant for the improvement of sustainable production of halophytes using salinized soils or brackish waters, as high value-added crops for commercial purposes.

1. Data

The sea knotgrass plants were produced in a greenhouse under different irrigation conditions (freshwater, 100, 200 and 300 mM of NaCl), and submitted to three consecutive harvests. Obtained biomass (above ground organs) were extracted with acetone, and the extracts were tested for *in vitro* antioxidant [radical-scavenging activity (RSA) of DPPH and ABTS, copper chelating activity (CCA) and ferric reducing antioxidant power (FRAP)] and anti-inflammatory (nitric oxide reduction on lipopolysaccharide-stimulated macrophages) properties. The results of half maximal inhibitory concentration (IC_{50}) are reported in Tables 1 and 2, for antioxidant and anti-inflammatory, respectively. For the same treatment, the RSA towards DPPH and ABTS, and CCA

Table 1
In vitro antioxidant activities of the acetone extract of sea knotgrass aerial parts irrigated with freshwater (approximately 0 mM of NaCl) and artificial saltwater with different NaCl concentrations (100, 200 and 300 mM of NaCl), for 3 repeated harvests. Results are expressed as IC_{50} values (μg/mL).

Assay	Harvest	0 mM NaCl	100 mM NaCl	200 mM NaCl	300 mM NaCl
DPPH	1st	nd	nd	nd	679 ± 13^{c}
	2nd	nd	nd	584 ± 29^{b}	–
	3rd	138 ± 2^{a}	664 ± 12^{c}	–	–
	BHT*	111 ± 1^{a}			
ABTS	1st	nd	nd	nd	705 ± 30^{c}
	2nd	704 ± 10^{c}	728 ± 37^{c}	nd	–
	3rd	279 ± 27^{b}	nd	–	–
	BHT*	140 ± 1^{a}			
CCA	1st	nd	nd	nd	nd
	2nd	nd	nd	nd	–
	3rd	560 ± 22^{b}	nd	–	–
	EDTA*	171 ± 9^{a}			
FRAP	1st	182 ± 5^{cd}	389 ± 15^{f}	216 ± 9^{de}	273 ± 17^{e}
	2nd	277 ± 10^{e}	233 ± 11^{de}	145 ± 6^{bc}	–
	3rd	54 ± 4^{a}	117 ± 8^{b}	–	–

-: Samples that did not survived until the harvest; nd: not determined (activity lower than 50% at 1000 μg/mL); *: positive control. Values represent the mean ± standard error of the mean (SEM) of four experiments (n = 4). For the same assay, , values followed by different letters (DPPH and ABTS: a–c; CCA: a–b; and FRAP: a–f) are significantly different at $P < 0.05$ (Tukey HSD test).

Table 2
In vitro anti-inflammatory activity of the acetone extract of *P. maritimum* aerial parts irrigated with freshwater (approximately 0 mM of NaCl) and artificial saltwater with different NaCl concentrations (100, 200 and 300 mM of NaCl). Results are expressed as IC_{50} values (μg/mL).

Harvest	0 mM NaCl	100 mM NaCl	200 mM NaCl	300 mM NaCl
1st	53.1 ± 2.1^{b}	52.8 ± 3.4^{b}	53.7 ± 2.0^{b}	51.4 ± 8.7^{b}
2nd	nd	nd	42.7 ± 5.5^{ab}	–
3rd	87.7 ± 1.6^{c}	nd	–	–
L-NAME*	27.6 ± 2.2^{a}			

-: Samples that did not survived until the harvest; nd: not determined (activity lower than 50%); *: positive control. Values represent the mean ± standard error of the mean (SEM) of four experiments (n = 4). Values followed by different letters (a–c) are significantly different at $P < 0.05$ (Tukey HSD test).

increased with the harvest. The same tendency was observed in FRAP, except on freshwater-irrigated plants that showed decreased activity from 1st to 2nd harvest, however decreasing in the 3rd harvest. The anti-inflammatory activity decreased with the harvest, and the lowest IC_{50} values were obtained on biomass from the 1st harvest, for all treatments. A detailed chemical profiling was performed by LC-UHRMS [1] and differences between treatments and harvests were analysed by PCA and PLC-DA statistics (Figs. 1–3). Striking differences on the chemical composition of statistically significant peaks tends to differ along with consecutive harvests and showed clear separation of salt concentration treatments disregarding the harvest sequence.

2. Experimental design, materials, and methods

2.1. Extraction

The dried biomass of aerial parts (leaves, stems and shoots) was extracted with pure acetone (1:40, w/v) in an ultrasonic bath [1]. The extracts were filtered (Whatman no. 4) and acetone was removed by rotary evaporation. The dried extracts were weighed, resuspended in methanol at 10 mg/mL, and stored at −20 °C.

2.2. Radical-scavenging activity (RSA) on DPPH and ABTS

The DPPH and ABTS RSA of the extracts at different concentrations (10–1000 µg/mL) was performed as reported earlier [2]. Differences in absorbance were measured in a microplate reader (Biotek Synergy 4). Butylated hydroxytoluene (BHT) was used as standard at concentrations equal to those of the samples. Results were expressed as an inhibition percentage, comparative to a control containing methanol instead of the sample, and as half maximal inhibitory concentration (IC_{50} values, µg/mL).

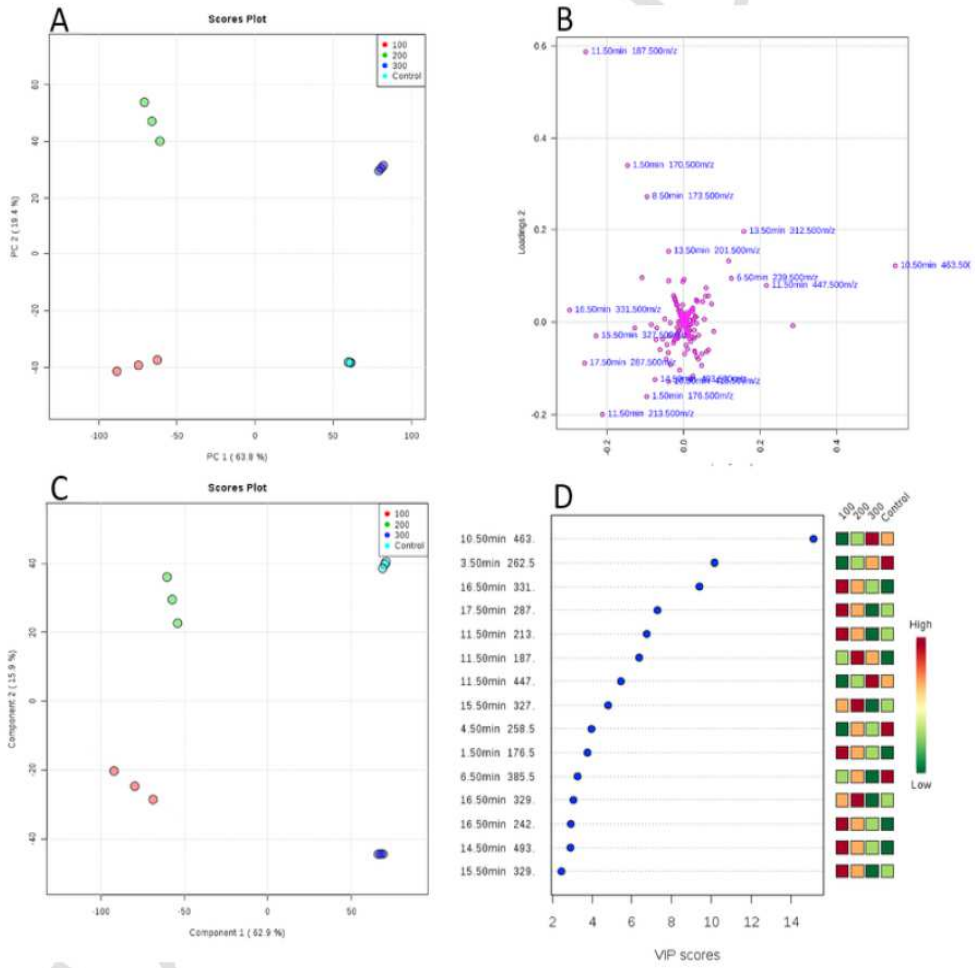

Fig. 1. (A) PCA scores plot based on UHPLC–MS data showing separation amongst samples from different irrigation conditions (0 mM - Light Blue; 100 mM – Red; 200 mM – Green; and 300 mM - Deep Blue) for the 1st harvest, together with their respective 95% confidence regions. The explained variances are shown in brackets (PC 1 and PC 2: 63.8 and 19.4%, respectively). **(B)** The corresponding loadings scatter plot showing the compounds (represented by their retention times [RT]) that are correlated to separation in scores plot. **(C)** PLS-DA of metabolites between groups described above (component 1 and component 2: 62.9 and 15.9%, respectively). **(D)** Variables important in projection (VIP) scores of 15 top contributors (shown as RT) to PLS-DA component 1.

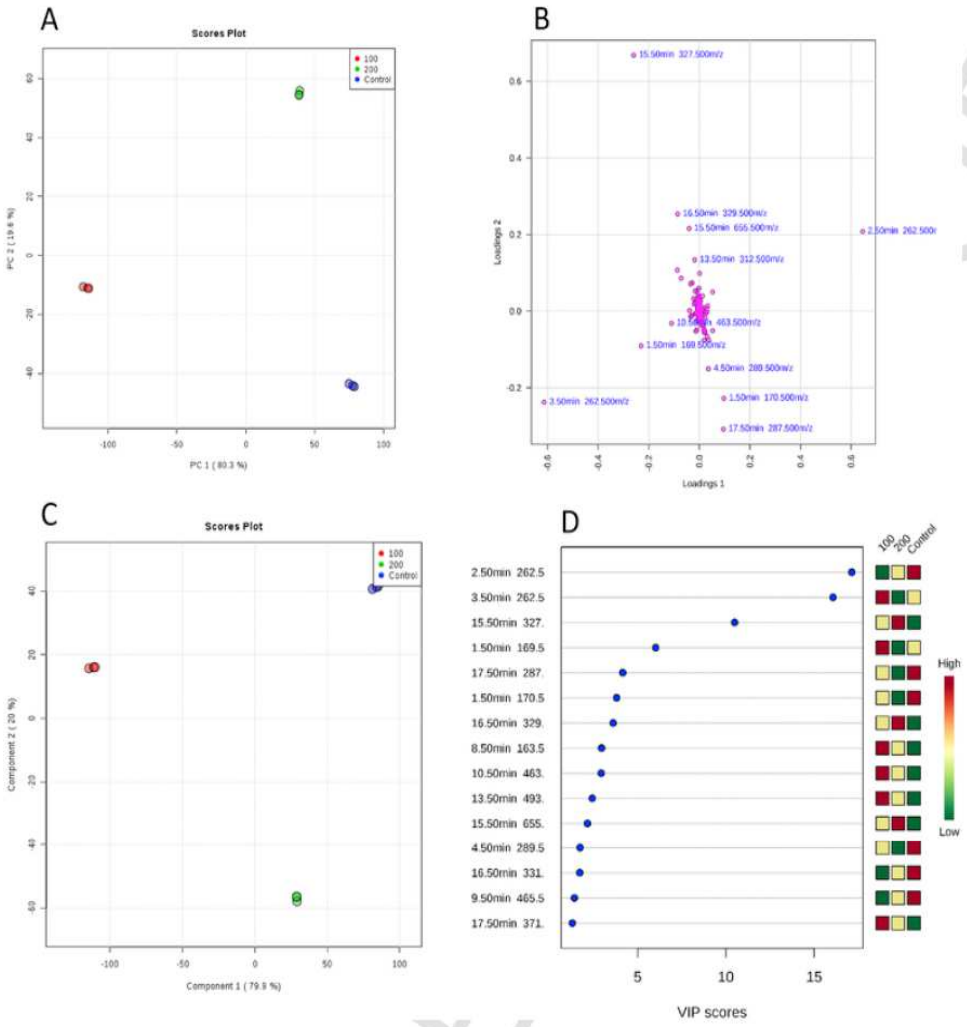

Fig. 2. (A) PCA scores plot based on UHPLC–MS data showing separation amongst samples from different irrigation conditions (0 mM - Deep Blue; 100 mM – Red; and 200 mM – Green) for the 2nd harvest, together with their respective 95% confidence regions. The explained variances are respectively 80.3 and 19.6% for PC 1 and PC 2. **(B)** The corresponding loadings scatter plot showing the compounds (shown as RT) that are correlated to separation in scores plot. **(C)** PLS-DA of metabolites between groups described above (component 1 and component 2 are 79.9 and 20%, respectively). **(D)** Variables important in projection (VIP) scores of 15 top contributors (presented as RT) to PLS-DA component 1.

2.3. Ferric reducing antioxidant power (FRAP)

The extracts' capacity to reduce Fe^{3+} (at concentrations amongst 10–1000 µg/mL) was evaluated as described by Rodrigues et al. [2]. An increase in the absorbance at 700 nm in the reaction mixture indicates an increased reducing power of the samples (Biotek Synergy 4). Results were calculated as a percentage in relation to the standard (BHT, 1000 µg/mL), and as IC_{50} values (µg/mL).

2.4. Metal chelating activity on copper (CCA)

The CCA of the extracts (at concentrations varying between 10 and 1000 µg/mL) was assayed as depicted before [2]. The color switch was measured on a microplate reader (Biotek Synergy 4), and ethylenediaminetetraacetic acid (EDTA) was applied as the positive control at the identical concentrations of the extracts. Results were presented as an inhibition percentage comparatively to a control using methanol as substitute of the sample, and as IC_{50} values (µg/mL).

2.5. Cell culture and cell viability

RAW 264.7 cells were grown in RPMI 1640 culture medium complemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL)/streptomycin (50 µg/mL) and were kept at 37 °C in moistened en-

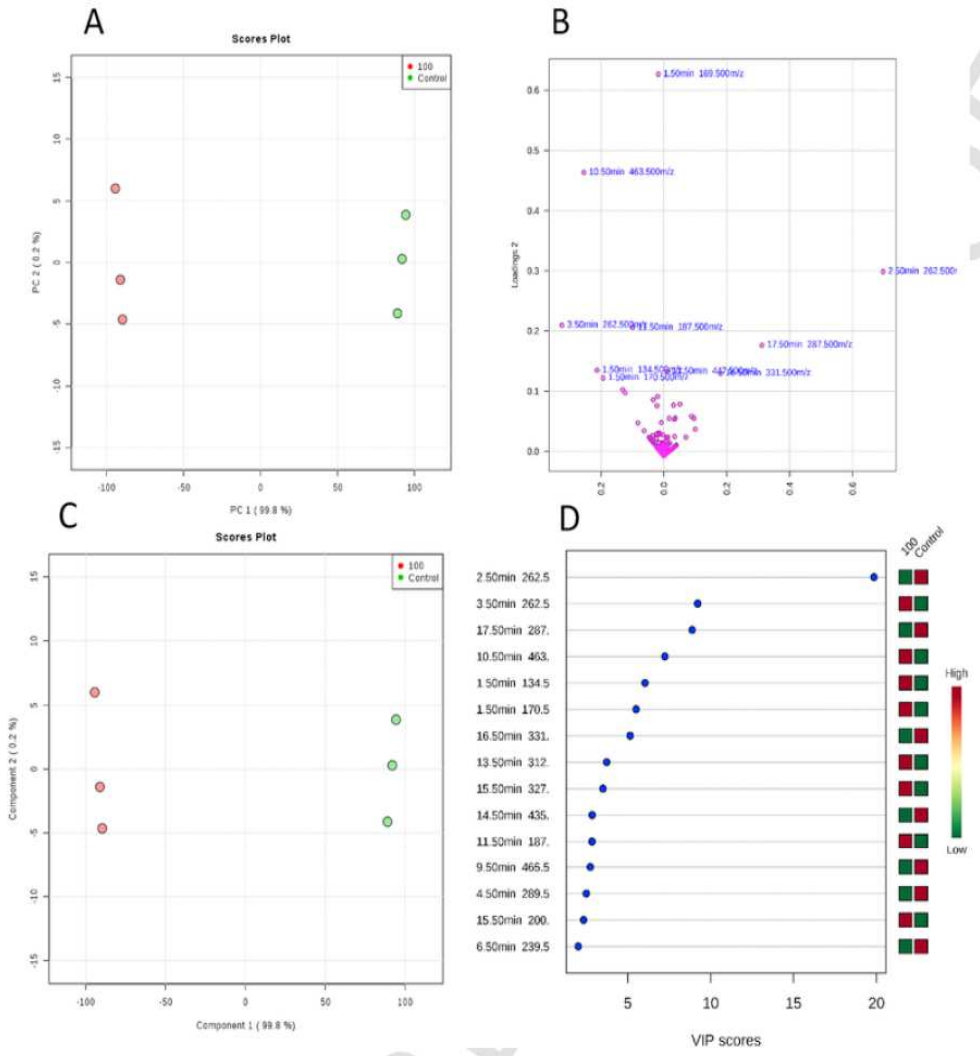

Fig. 3. (A) PCA scores plot based on UHPLC–MS data showing separation samples from different irrigation conditions (0 mM - Green; and 100 mM – Red) for the 3rd harvest, together with their respective 95% confidence regions. The explained variances are 99.8 and 0.2% for PC 1 and PC 2, correspondingly. **(B)** The corresponding loadings scatter plot showing the compounds (represented by their RT) that are correlated to separation in scores plot. **(C)** PLS-DA of metabolites between groups described above (component 1 and component 2: 99.8 and 0.2%, respectively). **(D)** Variables important in projection (VIP) scores of 15 top contributors (displayed as RT) to PLS-DA component 1.

vironment with 5% CO_2. Cells were seeded at a concentration of 1×10^4 cells/well, in 96-well microplates. After 24h of incubation, the extracts were added at concentrations from 3 to 100 µg/mL, and incubated for 24h. Cells treated with the vehicle (0.5% DMSO, v/v) were used as negative control, and cell viability was assessed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric test [3]. Results were calculated as a percentage of cell viability, in comparison with the control cells.

2.6. In vitro anti-inflammatory assay

The samples were tested for their capacity to decrease nitric oxide (NO) production in RAW 264.7 macrophages [3]. Cells were plated at 2.5×10^5 cells/well in 96-well plates and left to adhere overnight. Then, non-toxic concentrations of the extracts (>80% of cell viability) were incubated in serum- and phenol-free culture medium, with 100 ng/mL of LPS, for 24h. The cellular NO production was evaluated by the Griess method [3]. Results were expressed as a percentage (%) of NO production comparing to a control cells containing DMSO (0.5%, v/v), and as IC_{50} values (µg/mL).

2.7. Liquid chromatography/ultra-high-resolution mass spectrometry (LC-UHRMS)

Samples were pre-treated using solid phase extraction as follows: 100 mg of the extracts were suspended in 1 mL of 0.2% formic acid in purified water (HLP10Uv, Hydrolab, Gdańsk). Next, the suspension was loaded to the C18 Sep-Pak cartridges (1

cm^3, 360 mg, Waters Corp., Milford, MA) and washed with 0.5% methanol to remove carbohydrates and then with 80% methanol to elute phenolics. The phenolic fraction was re-evaporated, dissolved in 1 mL of 0.2% formic acid in 80% aqueous methanol, centrifuged for 5 min at 23 000×*g*, and filtered through 0.22 μm syringe filters (mix cellulose esters, Carl Roth, Karlsruhe, Germany) before LC-MS analysis (stored at −20 °C before analysis for no longer than 3 days). All analyses were performed in triplicate for three independent samples.

Liquid chromatography (LC) – electrospray ionization (ESI)-QTOF-MS was carried out using Thermo Dionex Ultimate 3000 RS system consisting of a binary pump system, sample manager, column manager and a DAD detector (Thermo Fischer Scientific, Waltham, MA), coupled to a Bruker Compact quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Billerica, MA). Separations were performed on a Kinetex C18 column (2.1 × 100 mm, 2.6 μm, Phenomenex, USA), with mobile phase A consisting of 0.1% (v/v) formic acid in water and mobile phase B containing 0.1% (v/v) formic acid in acetonitrile. A linear gradient from 1% to 60% phase B in phase A over 20 minutes was used to separate phenolic compounds. The flow rate was 0.4 mL/min, and the column was held at 30 °C. Mass spectra were acquired in negative-ion mode with 5 Hz frequency over a mass range from m/z 100 to 1500. Operating settings of the ESI ion source were as follows: capillary voltage 3 kV, dry gas flow 6 L/min, dry gas temperature 200 °C, nebulizer pressure 0.7 bar, collision radio frequency 700.0 V, transfer time 100.0 μs, and pre-pulse storage 7.0 μs. Ultrapure nitrogen was used as drying and nebulizer gas, and argon was used as the collision gas. The collision energy was set automatically from 15 to 75 eV depending on the m/z of the fragmented ion. For calibration of the accurate mass measurements, we used sodium formate introduced to the ion source at the beginning and end of each separation via a 20 μL loop. After data acquisition, raw UPLC–QTOF-MS spectra (negative mode) were pre-processed using a ProfileAnalysis software (version 2.1, Bruker Daltonik GmbH, Germany). Parameters of ProfileAnalysis were used as follows: advanced bucket generation with retention time range of 0–20 min, mass range of 100–800 m/z, each bucket (spectral bins) was formed with 1 min and 1 m/z delta, 0.2 kernelizing value, without normalization, background subtraction, and time alignment. LC-MS analyses were processed with the Find Molecular Futures (FMF) function to create compounds (molecular features) with S/N- 3 for peak detection. Generated bucket table consisting of tR:m/z pairs and respective compound intensity was exported and uploaded to MetaboAnalyst program. Each obtained dataset was filtered and normalized to the sum of peak areas and mean-centered scaling.

Acquired spectra were processed with Bruker DataAnalysis 4.3 software. The quality of the isotopic fit was expressed by the mSigma-value. The matched peaks from SmartFormula3D were sent to MetFrag website for computer-assisted *in silico* fragmentation and identification of metabolite mass spectra. Additionally, we searched the web-based databases for potential matches to the detected compounds: the human metabolome database (http://www.hmdb.ca/), the BiGG database (http://bigg.ucsd.edu/), the PubChem database (http://pubchem.ncbi.nlm.nih.gov/), the MassBank database (http://www.massbank.jp), KEGG (www.genome.jp) and the Metlin database (http://metlin.scripps.edu).

2.8. Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM) of at least three repetitions. Significant differences were evaluated by analysis of variance (ANOVA) and by the Tukey HSD test ($P < 0.05$). Statistical analyses were made using the XLSTAT statistical package for Microsoft Excel (version 2013, Microsoft Corporation). The IC_{50} values were calculated by a sigmoidal fitting of the data (GraphPad Prism v. 5.0 program).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dib.2019.104357.

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[1] M.J. Rodrigues, I. Monteiro, C. Placines, V. Castañeda-Loaiza, S. Ślusarczyk, A. Matkowski, C. Pereira, P. Pousão-Ferreira, L. Custódio, The irrigation salinity and harvesting affect the growth, chemical profile and biological activities of Polygonum maritimum L, Ind. Crops Prod. 139 (2019) 111510 https://doi.org/10.1016/j.indcrop.2019.111510.

[2] M.J. Rodrigues, A. Soszynski, A. Martins, A.P. Rauter, N.R. Neng, J.M.F. Nogueira, J. Varela, L. Barreira, L. Custódio, Unravelling the antioxidant potential and the phenolic composition of different anatomical organs of the marine halophyte Limonium algarvense, Ind. Crops Prod. 77 (2015) 315–322 https://doi.org/10.1016/j.indcrop.2015.08.061.

[3] M.J. Rodrigues, K.N. Gangadhar, C. Vizetto-Duarte, S.G. Wubshet, N.T. Nyberg, L. Barreira, J. Varela, L. Custódio, Maritime halophyte species from southern Portugal as sources of bioactive molecules, Mar. Drugs 12 (2014) 2228–2244 https://doi.org/10.3390/md12042228.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1. General discussion

In this study, seven halophyte species from the Algarve coast (southern Portugal), namely *F. laevis* (sea-heath), *H. amplexicaulis*, *J. acutus* (spiny rush), *J. inflexus* (wire rush), *J. maritimus* (seaside rush), *L. algarvense* (sea lavender) and *P. maritimum* (sea knotgrass) were selected for their promising biological activities disclosed during an R&D project, and further explored as potential sources of bioactive ingredients with therapeutic, cosmetic and/or nutritional applications. The work started with a preliminary screening of the bioactive properties of the mentioned species, that included the determination of the *in vitro* antioxidant, neuroprotective, antidiabetic and anti-melanogenic activities of dichloromethane and methanol extracts (Chapter 2). The obtained results were evaluated, and the most promising species and biological activities were further studied. The work was divided into chapters, each one corresponding to a halophyte species or genus. This chapter aims at a general discussion of all the work, through the comparison of the obtained results for each species or genus. The selection of the extraction solvents was made according to the potential application of each species or genus: when selected as potential beverages, infusions and decoctions were chosen, in turn for food supplements, nutraceutical or cosmetic applications low-toxicity solvents were preferred, namely acetone, ethyl acetate, ethanol and water. When selected as a potential source of compounds for the pharmaceutical industry, methanol and dichloromethane were selected. In some studies, and despite not being part of the thesis plan, molecular docking (*in silico*) studies were performed in collaboration with Adriano Mollica (Department of Pharmacy, University "G. D'Annunzio" of Chieti-Pescara, Chieti, Italy), Ramin Ekhteiari Salmas and Serdar Durdagic (Computational Biology and Molecular Simulations Laboratory, Department of Biophysics, School of Medicine, Bahcesehir University, Istanbul, Turkey) to better understand the biological effects of the identified molecules, complementing and enabling the publication of the corresponding papers.

6.1.1. Phytochemical profile

The phytochemical characterization of the extracts included determining its main bioactive compounds, such as phenolic compounds, one of the most widespread plant components group with therapeutic applications (Balasundram et al. 2006). In this sense, the extracts of sea lavender and sea knotgrass were estimated for their total contents in phenolics (TPC), flavonoids (TFC), tannins, hydroxycinnamic acids, anthocyanins, and flavone and

flavonols. However, only the TPC and TFC will be discussed, since they are the common assay performed for both species. Natural extracts are considered rich in phenolics when their TPC is higher than 20 mg of gallic acid equivalents (GAE)/g DW (Kähkönen et al. 1999). This was the case of the methanol extracts from all the plant organs of sea lavender (54 – 228 mg GAE/g), of the infusions and decoctions of the sea lavender flowers (179 – 191 mg GAE/g DW), and also of the aerial parts of the sea knotgrass extracted with ethyl acetate, isopropanol, ethanol, acetone, water at RT, and water at 100ºC, and their mixtures with 50% of water, which varied amongst 37.3 and 241 GAE/g DW. Regarding the TFC, the same classification as for TPC will be applied, but in terms of rutin equivalents (RE). Similarly to TPC, all methanol extracts from all sea lavender anatomical organs had high contents in flavonoids (44 – 236 RE/g DW), as well as flowers infusions and decoctions (96.0 – 106 RE/g DW). However, only a few sea knotgrass extracts presented high TFC according to the adopted classification, such as those prepared with ethyl acetate, 50% ethyl acetate, 50% isopropanol, ethanol, acetone and 50% acetone (21.8 – 49.0 RE/g DW), being that acetone and ethyl acetate extracts had the highest levels. In this sense, both sea lavender and sea knotgrass had markedly high levels of total phenolics and flavonoids being a potential source of these bioactive molecules. When comparing the two species, all extracts from sea lavender flowers had phenolic and flavonoid contents comparable to that of the aerial parts of sea knotgrass extracted with acetone. The different accumulation pattern of phenolics and flavonoids found in sea lavender organs is probably linked to the different roles of phenolics in the plants' interaction with their environment. Phenolic compounds can accumulate in different plant tissues due to the different physiological roles of each organ: they can act as UV filters (flavonoids in particular) protecting large radiation-exposed areas, as phagodeterrents against herbivores, or contribute to pigmentation that attracts pollinators (Hutzler et al. 1998; Harborne and Williams 2000; Petersen and Simmonds 2003). Besides, the difference found amid the sea knotgrass extracts on TPC and TFC is probably related to the use of different solvents that have different capacities in extracting the phenolic compounds, which also vary with the species and biomass characteristics (Buhmann and Papenbrock 2013; Qasim et al. 2016).

Phenolic compounds in plants are an extremely diverse group of molecules, thus a more detailed polyphenolic profile of the extracts was established by high-performance liquid chromatography (HLPC) for sea lavender and by ultra-high-resolution mass spectrometry (UHRMS) and gas chromatography and mass spectrometry (GC-MS) for sea knotgrass. This latter technique also allowed the identification of compounds belonging to other biochemical classes (alkanes/alkenes, fatty acids, acylglycerols, saccharides, alcohols and phytosterols). A

great diversity of phenolics was found in both species, and the main molecules detected in the sea lavender samples were gallic acid (in the methanol extract, infusion and decoction), salicylic and gentisic acids (in the infusion and decoction), and catechin (in the methanol extract). As expected, the combination of sea lavender flowers with green tea increased the phenolics diversity of the resulting beverages with p-hydroxybenzoic, caffeic and syringic acids. In turn, the main phenolics in the sea knotgrass extracts were myricitrin (methanol, dichloromethane and acetone), a myricetin derivative (methanol and dichloromethane extracts) and catechin (acetone extract). Regarding the greenhouse produced sea knotgrass plants, myricetin and quercetin glycosides were the most abundant phenolics in the obtained extracts, and some compounds allowed the distinction amongst the different treatments. For example, hyperoside (quercetin-3-O-galactoside) allowed differentiating between salinity treatments within the 1st and 2nd harvests, being most relatively abundant at 100 mM. Caffeic acid 3-sulfate discriminated between harvests, in the same irrigation treatment (it was detected at higher amounts on the 1st harvest and dropped on the 2nd and 3rd harvests). In turn, the flavonoids quercetin, apigenin, luteolin, and naringenin, and its glycoside derivatives were the main compounds detected in the greenhouse cultivated sea lavenders, and their presence varied within the different plant organ, specific irrigation salinity, wild or cultivated plants. The flowers were the richest plant organ in the above-mentioned compounds, and apigenin and luteolin contents increased with the irrigation salinity, whereas myricetin decreased. Quercetin was only detected in wild plants, while eriodictyol was only identified in cultivated sea lavenders. Moreover, galloyl glucose and galloyl hexoside derivatives were only present in flowers of freshwater-irrigated plants.

Phenolics are generally produced by plants as a result of environmental interactions, and thus their distribution amongst the diverse tissues and organs is usually related to their function (Kutchan 2001; Lattanzio et al. 2006). For example, phenolic acids were mostly detected in sea lavender extracts, mainly hydroxybenzoic acids that are described to exhibit allelopathic effects (through the inhibition of others plant species growth), pathogenic fungi inhibition functions (limiting the spore germination and hyphal growth), besides being produced in response to plant cutting or herbivory (Dix 1979; Ananthakrishnan 1990; Hättenschwiler and Vitousek 2000; Seal et al. 2004; Weir et al. 2004). Flavonoids were the main components of sea knotgrass, and have diverse functions, including pigmentation roles to attract pollinators to increase the fertilization rates, and animals to eat the fruits and spread the seeds (Mol et al. 1998; Pichersky and Gang 2000). 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). Thus, this type of compounds is very important in halophyte plants that live in environments with high levels of irradiation, therefore they act most probably as an antioxidant defence mechanism (Jithesh et al. 2006; Ksouri et al. 2010). Moreover, the synthesis of phenolics and flavonoids is modulated by variations on biotic and/or abiotic stresses (*e.g.* salinity, drought, UV radiation and/or herbivory), hence, their levels may rise and/or decrease when plants are exposed to environmental stresses, such as increasing irrigation salinity and harvesting (mimics herbivory) (Ksouri et al. 2007).

Additionally, all the identified molecules have multiple beneficial properties for human health, for example as antioxidants (Foti 2007; Dai and Mumper 2010), anti-inflammatory (gallic and salicylic acids, catechin, and flavonoid glycosides; Comalada et al. 2005; Rains et al. 2011; Domitrović et al. 2015; Vallverdú-Queralt et al. 2015), hypoglycemic (catechin; Yilmazer-Musa et al. 2012), anti-hyperpigmentation (catechins and flavonoid glycosides; Sato and Toriyama 2009; Chaiprasongsuk et al. 2016), and neuroprotective (gallic, gentisic and salicylic acids; Szwajgier 2015). Most of the phenolic compounds identified in sea lavender and sea knotgrass were already described as promising and important bioactive molecules with high biotechnological potential as bioactive ingredients for the development of innovative products with health-promoting properties for the cosmetic, food and pharmaceutical areas.

6.1.2. Biological activities

The seven halophytes species used in the initial screening were evaluated by several *in vitro* assays targeting antioxidant (DPPH, ABTS and NO radical scavenging, copper and iron chelating, and ferric reducing), antidiabetic (α-glucosidase and α-amylase inhibition), neuroprotective (inhibition of acetyl- and butyrylcholinesterase) and anti-hyperpigmentation (inhibition of tyrosinase) activities (Chapter 2). The species with the most promising results were *Juncus* species (Chapter 3), sea lavender (Chapter 4) and sea knotgrass (Chapter 5). These species were further explored by using additional assays, including cell-based methods. For the sea knotgrass, other skincare-related activities were evaluated, namely anti-wrinkles, anti-acne and anti-microbial; however, the extracts were not active and therefore those results will not be included in the general discussion. A comparative analysis and discussion of the bioactivities found in these plants is presented below.

6.1.2.1. *In vitro* antioxidant properties

Antioxidants are molecules that can stabilize or deactivate free radicals before cellular damages occur, maintaining the main cellular functions, or chelate redox metals to prevent ROS generation (Kohen and Nyska 2002). Therefore, antioxidants can protect the organism from oxidative stress-related ailments, including coronary diseases, cancer, age-related degenerative brain disorders, T2DM, and chronic inflammation (Bogdan Allemann and Baumann 2008; Kusumawati and Indrayanto 2013; Sindhi et al. 2013; Kumar et al. 2015). Besides, antioxidants are often used to preserve foods and beverages, as well as cosmetic products, preventing deterioration and increasing the products' shelf life (Pokorný 1991; McCarthy et al. 2001; Jung et al. 2017). Nowadays we witness the increasing awareness of the importance of replacing synthetic antioxidants by natural ones, due to the lower toxicity and side effects of the latter compounds. In this sense, and in view of the growing consumers' needs and preferences for natural and safer products with health-promoting properties, there is a emergent demand to use medicinal plants as a source of novel ingredients for food and cosmetic formulations, such as halophytes (Gruenwald 2009; Sindhi et al. 2013).

Within this context, the evaluation of the *in vitro* antioxidant capacity of the selected halophyte species was performed by different complementary antioxidant assays to assess diverse reactive species and their different antioxidant mechanisms (Badarinath et al. 2010; Niki 2010). This included radical-based assays (RSA on DPPH, ABTS, NO, $O_2^{\bullet-}$ and $^{\bullet}OH$ radicals), metal-related methods (FRAP and metal chelation of iron ICA and copper), and lipid peroxidation tests [initial (FTC) and later (TBA) stages]. Amongst all extracts of sea lavender, methanol extract from flowers had the highest activity towards DPPH and ABTS radicals (IC_{50} values: 0.09 and 0.27 mg/mL, respectively), on copper chelation (IC_{50} = 0.29 mg/mL) and FRAP (IC_{50} = 0.01 mg/mL) comparing to infusions and decoctions. However, the latter extracts had a higher capacity to chelate iron (IC_{50} values: 0.47 – 0.62 mg/mL). Sea lavender had a similar antioxidant capacity activity to that of green tea, and when combined in a mixed herbal beverage, synergistic interactions were observed on the RSA towards $^{\bullet}OH$ and on the anti-lipid peroxidation capacity. Moreover, compared to the cultivated sea lavenders, the most active extracts were those from the wild plants; however, extracts obtained from cultivated plants also had a high antioxidant capacity, especially flowers from those irrigated with 300 mM of NaCl (IC_{50} values: 0.12 – 0.40 mg/mL).

Regarding the sea knotgrass, the aerial parts showed higher RSA towards the DPPH and ABTS radicals, reducing capacity (FRAP) and copper chelation properties (IC_{50} values: 0.03,

0.14, 0.05 and 0.29 mg/mL, respectively). These results were better than those obtained in extracts prepared with biomass from cultivated plants. Furthermore, the extracts from sea knotgrass plants irrigated with freshwater were more active than those from plants irrigated with artificial saltwater (100 mM NaCl), especially at the 3rd harvest, on the RSA towards DPPH and ABTS, and FRAP (IC_{50} values: 0.14, 0.28, 0.56 and 0.05 mg/mL).

Amongst the *Juncus* species, the extracts made with roots' biomass had in general higher activity than those made with aerial parts, and the spiny rush extracts had the lowest IC_{50} values for all the antioxidant methods (0.17 – 0.63 mg/mL). Globally, the sea knotgrass leaf extracts were more effective towards DPPH, ABTS and $O_2^{\bullet-}$ radicals than the spiny rush and sea lavender flower ones, but these latter were more active on FRAP. Both sea lavender and sea knotgrass showed similar copper chelating activities.

The antioxidant activity displayed by extracts from halophyte species is generally attributed to their high content in phenolic compounds (Ksouri et al. 2012), and the same association was found in this work for both sea knotgrass and sea lavender. The presence of individual phenolics and their quantities may also explain this high activity, namely the phenolic acids detected in sea lavender and flavonoids in sea knotgrass. Taken together, the data contained in the previous chapters disclosures the potent *in vitro* antioxidant properties of these halophyte species, suggesting them as an important source of antioxidants, contributing to the prevention of oxidative stress-related situations or as food or cosmetics preservatives.

6.1.2.2. *In vitro* anti-inflammatory properties

The production of inflammatory mediators is common in several human diseases, such as cancer, diabetes, cardiovascular disorders, AD, rheumatoid arthritis, Crohn's disease, ulcerative colitis, and also in the skin ageing process (Blasko et al. 2004; Libby 2007; Holmes et al. 2009). Thus, targeting and reducing the production of the main inflammatory mediators became an important approach to prevent and treat chronic inflammatory-related disorders (Hofseth 2008). *In vitro* cultured macrophages are frequently used as an inflammation model, and if stimulated with LPS, which is an endotoxin produced by Gram-negative bacteria, they produce chronic inflammatory mediators, namely nitric oxide (NO) (Martich et al. 1993; Kubes 2000; Joo et al. 2014). In this model, a reduction of the NO macrophage production suggests an anti-inflammatory potential of the tested sample (Joo et al. 2014). Since the genera *Limonium* (Felter and Lloyd 1898) and *Polygonum* (Fan et al. 2011; Takasaki et al. 2011), are described in traditional medicine with anti-inflammatory properties, the extracts of sea lavender and sea

knotgrass were tested for their potential to reduce NO production in LPS-stimulated macrophages. Both species showed a significant anti-inflammatory potential, through the reduction of NO production by RAW 264.7 macrophages. The sea knotgrass acetone extract from aerial parts was the most effective, with the lowest IC_{50} value (22 μg/mL) comparable to that obtained with the positive control (L-NAME). However, the leaf dichloromethane extract of the sea knotgrass and the infusion and decoction from sea lavender's flowers also showed a relevant activity, with similar IC_{50} values (46.3 – 48.5 μg/mL). Moreover, the extracts from cultivated sea knotgrass also had anti-inflammatory properties, similar amongst all the salinity treatments for the first harvest (IC_{50} values: 51.4 – 53.7 μg/mL).

Phenolic compounds are described as a promising alternative to treat inflammatory-related diseases (Sergent et al. 2010), and the main phenolics identified in sea lavender's flowers extracts have anti-inflammatory assets, namely apigenin, salicylic, gallic, ferulic and coumaric acids (Kroes et al. 1992; Lee et al. 2007; Pragasam et al. 2013; Zhu et al. 2014), as well as myricitrin, catechin and monogalloylhexose (structure similar to β-glucogallin; Chang et al. 2013; Domitrović et al. 2015), which were identified in the sea knotgrass acetone extract. Moreover, compounds from different biochemical classes were also identified in the sea knotgrass'es leaves dichloromethane extract, and are possibly related to its anti-inflammatory capacity, namely phytosterols (β-sitosterol and stigmasterol), fatty acids (FA; linoleic and oleic acids) and one FA-derived alcohol (1-octacosanol) (Ren and Chung 2007; Vassiliou et al. 2009; Gabay et al. 2010; Loizou et al. 2010; Oh et al. 2010; de Oliveira et al. 2012). Overall, these results suggest that the extracts from sea lavender and sea knotgrass could be beneficial in inflammation-related disorders, as they can reduce NO production in mammalian macrophages.

6.1.2.3. *In vitro* antidiabetic properties

Diabetes mellitus is a metabolic disorder that comprises a high blood glucose level, called hyperglycaemia, and affects more than 350 million people worldwide (WHO 1999; Mathers and Loncar 2006; Danaei et al. 2011; Yarchoan and Arnold 2014). About 90% of all cases are T2DM that is often treated by inhibition of carbohydrate-hydrolysing enzymes, such as α-glucosidase and α-amylase, which reduces the breaking down of starch into sugar monomers, contributing to decrease the postprandial blood glucose levels (Kwon et al. 2007). Several *Polygonum* species are used in traditional medicine to treat diabetes, but there is no information regarding the antidiabetic potential of *Limonium* genus. The sea lavender infusion

and decoction showed a high microbial α-glucosidase inhibitory capacity (IC_{50} values = 0.05 and 0.04 mg/mL, respectively), higher than the activity displayed by green tea (0.12 mg/mL), which is often described as having antidiabetic properties (Atoui et al. 2005). However, the sea knotgrass methanol extract from roots and leaves were more efficient inhibitors of microbial α-glucosidase, with IC_{50} values of 0.02 and 0.03 mg/mL, respectively. Furthermore, only the sea knotgrass'es leaves dichloromethane extract was able to inhibit mammalian α-glucosidase (IC_{50} = 2.53 mg/mL). The extracts from both plants had a higher inhibitory capacity toward the enzymes than the positive control used, acarbose, which is a clinically used inhibitor of both α-glucosidase and α-amylase. However, none of the species showed the capacity to inhibit α-amylase.

Phenolic compounds can inhibit carbohydrate-hydrolysing enzymes like α-glucosidase (Krentz and Bailey 2005; Kwon et al. 2007; Bhandari and Ansari 2008; Asgar 2013), and therefore, the phenolic-rich extracts, such as the sea knotgrass and sea lavender ones, may be a source of inhibitors of those enzymes. For example, gallic, salicylic and gentisic acids, which were the main components of the sea lavender infusion and decoction, have antidiabetic properties like hypoglycaemic or digestive enzymes inhibitory activity (Peungvicha et al. 1998; Rena and Sakamoto 2014). Also, the phytosterols β-sitosterol and stigmasterol, and the unsaturated FA linoleic and oleic acids, which were the major constituents of the sea knotgrass'es leaves dichloromethane extract, are described as more active α-glucosidase inhibitors than the standard inhibitor acarbose (Ortiz-Andrade et al. 2007; Lean Teik et al. 2013; Su et al. 2013). These data suggest that the extracts from these two halophytes could be useful in managing T2DM by inhibiting dietary carbohydrate-hydrolysing enzymes and thus reducing blood glucose levels. Moreover, hyperglycaemia states promote metabolic alterations that contribute to the occurrence of oxidative stress and chronic inflammation, which in turn leads to diabetes-associated complications (Mai and Chuyen 2007; American Diabetes Association 2010; Vikram et al. 2014). Thus, therapies combining natural antioxidant and anti-inflammatory elements, such as sea lavender and sea knotgrass extracts, can help prevent and/or manage diabetes-associated complications (Ruhe and McDonald 2001; Devasagayam et al. 2004; Fardoun 2007; Akash et al. 2013).

6.1.2.4. *In vitro* neuroprotective properties

AD is the most common type of dementia in the elderly (generally after 65-years old) and is considered as the 6th leading cause of death in developed nations (Alzheimer's Association 2015). One of the main hallmarks of AD is the reduction of the levels of the neurotransmitter ACh that causes insufficient transmission of the neurological impulse, leading to difficulties in muscle contraction, memory and learning capabilities (Houghton et al. 2006; de Souza 2011). AChE and BuChE enzymes are responsible for the ACh hydrolysis, thus their inhibition results in increased ACh levels that benefit the cognitive function and slow down disease progression, contributing to the amelioration of the symptoms, thus improving the patients' quality of life (Tariot et al. 2000; Ballard 2002; Greig et al. 2005; Huang and Mucke 2012; Alzheimer's Association 2015; Kumar et al. 2015).

In this context, sea lavender, sea knotgrass and *Juncus* species were evaluated for their capacity to inhibit AChE and BuChE. Sea lavender infusion and decoction (IC_{50} values: 0.22 – 0.39 mg/mL), and sea knotgrass methanol (IC_{50} values: 0.17 – 0.27 mg/mL) extracts showed the highest AChE inhibition. Although generally less active towards BuChE, the extracts were also capable to inhibit this enzyme (sea lavender, IC_{50}: 0.84 – 0.96; sea knotgrass, IC_{50} = 0.61 – 0.62 mg/mL). In the case of sea lavender, the anticholinesterase activity synergistically increased when infusion and decoctions were made in combination with green tea.

Some particular phenolic acids present in sea lavender (gallic, vanillic, caffeic, gentisic and salicylic acids) and flavonoids found in the sea knotgrass extracts (quercetin-3-O-glucoside, avicularin, quercitrin and quercetin-3-O-glucuronide) have strong *in vitro* cholinesterase inhibitory capacities (Jung and Park 2007; Khan et al. 2009; Szwajgier 2015). Regarding *Juncus* species, only the extracts from spiny rush displayed cholinesterase inhibition towards BuChE, especially the leaves (IC_{50} = 0.66 mg/mL). This extract was fractionated, and a bioactive compound was isolated and identified as the phenanthrene juncunol (1,7-dimethyl-5-vinyl-9,10-dihydrophenanthren-2-ol). This compound was further tested on both enzyme- and cell-based assays, showing IC_{50} values of 758 and 940 μM for BuChE and AChE, respectively. Moreover, IC_{50} values 6 to 8 times lower were obtained in the AChE cell-based systems (IC_{50} values: 117 – 158 μM). Overall, all the obtained data suggest that sea lavender, sea knotgrass and spiny rush extracts, and/or compounds from them, could be beneficial in managing neurodegenerative diseases, such as AD since they are able to inhibit cholinesterase enzymes and consequently decelerate disease progression and reduce the associated symptoms.

Due to high lipid content, high oxygen metabolism and reduced antioxidant levels of the brain, oxidative stress is being linked to the development and progression of neurodegeneration (Uttara et al. 2009). Thus, based on the promising results obtained with the inhibition of cholinesterases by the sea knotgrass, methanol and dichloromethane extracts were further explored for their *in vitro* protective effects on H_2O_2 induced-cytotoxicity on human neuroblastoma SH-SY5Y cells. The co-application of sea knotgrass'es leaves and roots methanol extracts and H_2O_2 significantly increased the cellular viability up to 20% comparing to the cells only treated with H_2O_2, which suggests a protective effect of sea knotgrass against the oxidative stress-induced cytotoxicity. In fact, phenolics, particularly catechins found in the methanol extract, are likely involved in this antioxidant activity (Foti 2007; Dai and Mumper 2010).

Additionally, the inflammatory response to brain damage induces the production of pro-inflammatory mediators by microglial cells, which are the main players in neuroinflammation (Morales et al. 2014; Pasqualetti et al. 2015). Therefore, methanol and dichloromethane extracts from sea knotgrass were also appraised for their capacity to reduce the production of NO in an *in vitro* model of neuroinflammation (LPS-stimulated microglial N9 cells). The extracts displayed a high capacity to decrease NO production, with IC_{50} values of 4.17 – 9.95 µg/mL (methanol) and 14.2 – 18.0 µg/mL (dichloromethane). This capacity was higher than the one displayed by the used standard, L-NAME (IC_{50} = 39.3 µg/mL). The main components identified in the sea knotgrass extracts have been reported previously with *in vitro* anti-inflammatory activity, namely epigallocatechin, catechin, myricitrin and a quercetin derivative (Tipoe et al. 2007; Fan et al. 2011; Pereira et al. 2011).

Overall, compounds combining cholinesterase inhibition with antioxidant and anti-neuroinflammatory properties are considered effective in the prevention of brain oxidative damage and neuronal loss associated with neurodegeneration (Tabner et al. 2005; Uttara et al. 2009; Morales et al. 2014). Thus, the halophyte species under study can be considered promising sources of molecules with cognitive improvement properties.

6.1.2.5. *In vitro* anti-hyperpigmentation properties

An excessive melanin accumulation leads to diverse skin hyperpigmentation situations, such as melasma, freckles, post-inflammatory melanoderma and solar lentigo (Khan 2007; Hakozaki et al. 2015; Ribeiro et al. 2015; Tu and Tawata 2015). Tyrosinase is an essential

enzyme on the melanin biosynthetic pathway, and therefore, the use of tyrosinase inhibitors may prevent the excessive melanin accumulation (Khan 2007; Hakozaki et al. 2015; Ribeiro et al. 2015; Tu and Tawata 2015). Moreover, tyrosinase inhibitors are also useful to reduce the browning of fruits and vegetables in post-harvest processing (Khan 2007; Chang 2009). Hence, tyrosinase inhibitory ingredients are valuable for both the cosmetic and food industries to reduce skin hyperpigmentation situations and to preserve foods. Following this, the sea knotgrass extracts were evaluated for anti-hyperpigmentation potential by assessing its capacity to inhibit tyrosinase and to reduce melanin production in B16 melanoma cells. The acetone extract had an IC_{50} value of 64.1 μg/mL towards tyrosinase, and also a high capacity to reduce the melanin production on the B16 4A5 melanoma cells (IC_{50} = 77.7 μg/mL). It is known that phenolic compounds have protective effects on UV-mediated skin pigmentation, due to their antioxidant and UV-absorbing actions (Chaiprasongsuk et al. 2016). But besides that, the main phenolics found in this extract may be responsible for the *in vitro* depigmenting effects, namely catechins and its derivatives, monogalloyl-hexose (structurally similar to β-glucogallin) as well as proanthocyanins and its dimers (Sato and Toriyama 2009; Majeed et al. 2010; Tatsuno et al. 2012). In this sense, these results indicate sea knotgrass as a good source of compounds to manage skin hyperpigmentation conditions or to prevent food darkening.

6.1.3. *In vitro* toxicological assessment

Although the use of natural formulations in traditional medicine may indicate their safe consumption, it is still necessary to ensure their safety for their potential consumers. Initial toxicological evaluation of botanical ingredients can be assessed by simple, quick, consistent, sensitive, and cost-effective approaches, which may include a cytotoxicity evaluation on mammalian cell lines and the brine shrimp lethality assay (Logarto Parra et al. 2001; Carballo et al. 2002). In this context, the infusion and decoction of sea lavender were applied at 100 μg/mL for 72h to three mammalian cell lines, namely N9 (murine microglia), S17 (murine bone marrow stromal), HepG2 (human hepatocellular carcinoma), and on the brine shrimp (*Artemia salina*). The application of these samples rendered values of cellular and brine shrimp viabilities higher than 80%, which suggests that they are non-toxic. Additionally, the toxicological effects of commercial green tea infusion and decoction were also ascertained for comparison and displayed low toxicity on brine shrimp (> 80% of viability), HepG2 and N9 cells (> 70% of cell viability), but some toxicity towards S17 cells (< 30% of cell viability). The ethanol extracts

from the different plant organs of sea lavender produced in the greenhouse were also evaluated for their *in vitro* cytotoxicity on murine RAW 264.7 macrophages, human embryonic kidney (HEK) 293 and HepG2 cells. Generally, the extracts were non-toxic (cell viability > 80%), although, a few extracts showed some toxicity (cell viability: 67.4 – 78.2%), such as leaves and flower stems from the wild, leaves irrigated with aquaculture wastewater at 300 mM NaCl, and flower stems from plants irrigated with freshwater. Thus, the results obtained with sea lavender are promising mostly if compared to those obtained with the commercial green tea, suggesting that these beverages may be considered safe for consumption. Actually, these formulations are reported to be used in traditional medicine, which reinforces their likely safe consumption, but nonetheless, supplementary experiments on mammalian animal models should be performed.

6.1.4. Cytotoxic mechanism of juncunol

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver, mostly derived from chronic liver diseases, such as cirrhosis or hepatitis. HCC is an aggressive type of cancer with high metastatic potential and resistance to the current cytotoxic drugs, such as doxorubicin (Vizetto-Duarte et al. 2016), and there is, therefore, a great need to identify new leads for HCC chemotherapeutics. In this work, juncunol (a phenanthrene) was isolated and identified from the spiny rush, displaying *in vitro* selective cytotoxic activity towards human hepatocarcinoma (HepG2) cells.

Apoptosis induction is one of the main anti-tumour approaches, being a common mechanism of numerous anticancer drugs, as for example etoposide, cisplatin, and doxorubicin (Kaufmann and Earnshaw 2000). The apoptotic pathway is linked to mitochondrial membrane permeabilization by changes in the mitochondrial membrane potential, which may, in turn, contribute to mitochondrial impaired ROS formation and apoptotic process initiation (Kannan and Jain 2000; Simon et al. 2000; Vizetto-Duarte et al. 2016). Furthermore, cell cycle arrest also contributes to reducing tumour cells' growth and proliferation and induces apoptosis (Pietenpol et al. 2002). Considering this, the *in vitro* cytotoxic mechanism of juncunol was evaluated on HepG2 cells to elucidate its potential to induce apoptosis, decrease mitochondrial membrane potential, promote internal ROS production and influence the cell cycle. Results showed that juncunol increase apoptosis, decreases mitochondrial membrane potential and induces cell cycle arrest in the G0/G1 phase, in a concentration-dependent manner. In fact, other phenanthrenes have apoptotic-inducing abilities on cervical and hepatic tumour cells, namely

denbinobin, fimbriol B and 2,3,5-trihydroxy-4,9-dimethoxyphenanthrene, triptolide, cryptotanshinone and tylophorine (Kim et al. 2010; Yao et al. 2008; Yang et al. 2012), as well as the capacity to induce cell cycle arrest on colon and hepatic cancer cells, such as cryptotanshinone, tylophorine and tanshinone (Su et al. 2008; Wu et al. 2009; Park et al. 2014). The obtained results suggest juncunol as an appropriate candidate for a comprehensive evaluation of its anti-tumour mode of action on hepatocellular carcinoma.

6.1.5. Growth performance of cultivated species

Despite the high biotechnological and commercial potential of halophyte species (Ksouri et al. 2012; Petropoulos et al. 2018a,b) knowledge about their cultivation is limited and there is a need to develop agronomic systems for target species that are economically, socially and environmentally feasible. Due to their salt-tolerance capacity, halophytes can be cultivated in conditions where commercial glycophytes cannot, for example in greenhouse irrigated with saline water or in saline soils. Moreover, the levels of the bioactive secondary metabolites, such as phenolics, may be influenced by different agronomic systems, namely salt concentration of the irrigation water, fertilization, harvest time and cycle (Ventura et al. 2010, 2011, 2013).

Although the production of some halophytes has already been tested by several authors, including, for example, the species *Cakile maritima*, *Salicornia* sp., *Tripolium pannonicum*, *Atriplex* sp., *Plantago coronopus*, or *Sesuvium portulacastrum* (Ksouri et al. 2007; Boestfleisch et al. 2014; Slama et al. 2015; Bendaly et al. 2016; Slama et al. 2017), the cultivation of the species under study has not yet been tested. Results obtained during this thesis allowed the identification of the species sea knotgrass and sea lavender as promising sources of natural ingredients for cosmetics, food, and nutraceutical products. However, to supply the possible commercial demand, it is necessary to guarantee the supply of biomass, ensuring the sustainable production of these species. Thus, the greenhouse cultivation of the referred species was attempted for the first time.

The cultivation of the sea knotgrass was made in a greenhouse under different irrigation salinities, including approximately 0 (freshwater), 100, 200, 300 and 600 mM NaCl. The plants' aerial parts were harvested three times at treatment intervals of 6 weeks, and the produced plants were evaluated regarding their growth performance by determining the plant survival, plant height, leaf number and surface area, moisture and productivity (dried biomass). Afterwards, the aerial parts (leaves + stems) of cultivated plants were evaluated for their biological and

phytochemical properties. Plants did not survive when irrigated at the highest salinities of 300 and 600 mM, and died before the 2nd and 1st harvests, respectively. Plants irrigated with 200 mM of NaCl died before the 3rd harvest, whereas freshwater- and 100 mM-irrigated plants survived until the final cut. The increasing irrigation salinity and the multiple harvesting regime generally resulted in a decrease of the plant height, the leaf number and area, as well as in the moisture contents and overall productivity. Plants irrigated with freshwater that had the highest growth parameters (height: 35 cm; leaf number: 91.4; leaf surface area: 5.9 cm^2) and productivity (346 g/m^2 of dried biomass). In addition, the irrigation salinity and harvest regime influenced the *in vitro* antioxidant and anti-inflammatory activities and the chemical profile of extracts prepared with the obtained biomass (as described in sections 6.1.2.1 and 6.1.2.2).

Regarding sea lavender, plants were produced in a greenhouse under different irrigation salinities, including around 0 (freshwater), and saline aquaculture wastewater corresponding to 600 mM NaCl (whole water) and 300 mM NaCl (1:1 dilution with freshwater). The sea lavenders' aerial parts were harvested after 12-weeks of treatment, and the plant growth performance was determined by flower stems height and number, leaf number and surface area, moisture and dried yields. Then, the cultivated plants were separated in flowers, peduncles and leaves, for the biological activities' and metabolomic profile assessment.

Plants from all irrigation conditions survived until the harvest (12 weeks), however, plants irrigated with 600 mM NaCl failed to produce flowers and floral stems. Generally, the leaves had high moisture content and reduced dried matter, and freshwater-irrigated plants had the highest values (79.8 and 20.2%, respectively), as well as higher number (2.3) and height (36.4 cm) of floral stems, leaf number (54.7) and surface area (11.6 cm^2). The irrigation salinity decreased the moisture content (63.9 – 65.4%), the number and height of floral stems (1.3 and 14.3 cm, respectively), and the leaf number and surface area (11.1 – 21.1 and 5.2 – 8.5 cm^2, respectively). Furthermore, the irrigation conditions were correlated to the *in vitro* antioxidant and toxicological properties, and with the chemical profile of the obtained extracts (as detailed in sections 6.1.2.1 and 6.1.3).

When comparing both species, it is evident that they exhibited better growth and more relevant biological activities when irrigated with freshwater. However, plants irrigated with saline water also exhibited promising properties. It was also noticed that sea lavender can withstand a higher salt concentration in the irrigation solution (300 mM NaCl) than sea knotgrass (100 mM NaCl) and that both species were able to complete their life cycle at these concentrations (both produced flowers and viable seeds).

Although it is accepted that halophyte plants require salt to complete their life cycle, some studies on the halophytes' cultivation showed that many of these species (*e.g. Inula crithmoides*, *Plantago crassifolia* and *Medicago marina*) have better growth rates in the in non-saline conditions (Grigore et al. 2012). This can be explained by the fact that salinity is not the only limiting factor for halophytes, but also nutrients and water availability, indicating that the occurrence of these species in saline environments may be due to competition avoidance with non-salt tolerant plants. That is, due to their salt-tolerance capacity, halophyte plants gain the competition in saline conditions, but not in non-saline environments (Grigore et al. 2012). However, the salinity contributes to water deficit, reduced fresh weight, and plant growth, decreased photosynthesis, leaf injuries, nutritional deficiencies and physiological water stress (Koyro et al. 2008).

Overall, the data obtained in this work indicate that sea knotgrass and sea lavender are promising candidates for sustainable greenhouse cultivation using freshwater or moderate salinity water resources for irrigation (*e.g.* brackish water wells or diluted saline aquaculture wastewaters). Moreover, the interesting biological and chemical properties of the produced plants suggest them as valuable sources of ingredients for cosmetic, food, veterinarian and pharmaceutical industries.

6.2. Conclusions

In this work, seven halophyte species collected from the Algarve coast area were evaluated for their potential as sources of bioactive molecules and/or bioactive extracts with therapeutic, cosmetic and/or food applications. The main aim was to expand our limited knowledge on the biotechnological potential of Algarve's halophyte species. From those, three species showed the highest potential:

- **Spiny rush** (*Juncus acutus*; Chapter 3) – The infused fruits are traditionally mixed with barley grains to treat colds (El-Shamy et al. 2012). In this work, its leaves exhibited a high *in vitro* antioxidant, neuroprotective and anti-hepatocellular carcinoma properties. A main bioactive compound, juncunol, was isolated and identified with anti-cholinesterase and hepatocellular carcinoma apoptosis-inducing properties, indicating that this species is an effective source of ingredients for potential application in food, pharmaceutical, or nutraceutical products.

- **Sea lavender** (*Limonium algarvense*; Chapter 4) – Traditionally used in the form of infusions and decoctions to treat diarrhoea, inflammatory diseases or microbial infections (Felter and Lloyd 1898), displayed, particularly flowers, a high phenolic content (phenolic acids), and high *in vitro* antioxidant, anti-inflammatory, neuroprotective and antidiabetic properties Additionally, sea lavender could be cultivated in a greenhouse, conserving good antioxidant and chemical properties. when irrigated with moderate salinities. These results highlight its potential as a source of bioactive molecules for potential application on cosmetic, pharmaceutical or food industries, as food supplements or functional beverages while being cultivated in sustainable conditions.

- **Sea knotgrass** (*Polygonum maritimum*; Chapter 5) – Used in folk medicine for the treatment of dysentery, articular pain, inflammation and diabetes (Takasaki et al. 2001; Soumyanath 2005; Kawai et al. 2006; Fan et al. 2011; Bothon et al. 2013). Results revealed its high contents in flavonoids, and the high *in vitro* antioxidant, anti-inflammatory, neuroprotective, antidiabetic and anti-hyperpigmentation activities, which make this species a valuable source of ingredients with potential application as nutraceuticals, cosmetics or pharmaceutical ingredients. Also, sea

knotgrass showed great potential for greenhouse production, preserving relevant antioxidant, anti-inflammatory and chemical assets, when irrigated with low or moderate salinity.

After these considerations, we can assume that the main objectives of this work were successfully achieved:

- Objective 1: Evaluate the antioxidant, neuroprotective, antidiabetic and anti-hyperpigmentation activities of extracts made from different anatomical organs of halophyte species from the Algarve coast.
  - ✓ The halophyte species under study presented promising *in vitro* antioxidant (sea lavender, sea knotgrass and spiny rush), neuroprotective (sea lavender, sea knotgrass and spiny rush), antidiabetic (sea lavender and sea knotgrass) and anti-hyperpigmentation activities (sea knotgrass). These activities varied according to the different anatomical organs: the flowers extracts were the most active for sea lavender, while those from leaves of sea knotgrass and spiny rush had the highest biological properties.

- Objective 2: Characterize the phytochemical composition of the active extracts/fractions.
  - ✓ The phytochemical composition of the active extracts/fractions were characterized using different analytical methods, such as HLPC (for sea lavender and spiny rush), and UHRMS and GC-MS for sea knotgrass. The main bioactive compounds were identified, such as phenolic acids in sea lavender, flavonoids in sea knotgrass and juncunol (a phenanthrene) in the spiny rush.

- Objective 3: Select the most promising species and test their sustainable cultivation.
  - ✓ The most promising species (sea lavender and sea knotgrass) were successfully cultivated into a greenhouse, using fresh and saline water for irrigation. Both species showed better growth performance when irrigated with freshwater. The obtained extracts showed promising biological and chemical properties that varied with the irrigation conditions, including salinity and harvest regime for sea knotgrass, and salinity and plant organ for sea lavender.

- Objective 4: Elucidate the *in vitro* mechanism of action of juncunol, a selective antitumor molecule isolated from *Juncus acutus* L.
    - ✓ Juncunol exhibited an apoptosis-inducing capacity by reducing mitochondrial membrane potential, and cell cycle arrest in the G0/G1 phase.

In conclusion, the results of this work indicates that the three selected halophyte species are good candidates to be further explored as sources of ingredients for the food industry for example, as herbal beverages (sea lavender), for the pharmaceutical segment (sea lavender, sea knotgrass and spiny rush), or for the cosmetic industry (sea knotgrass). Moreover, sea lavender and sea knotgrass may be sustainably cultivated to provide biomass for commercial purposes. Hence, this thesis contributes to the promotion and valorisation of using these renewable and sustainable resources for industrial applications.

As future perspectives, several ideas came across. The sustainable cultivation techniques of selected species should be improved, in order to guarantee the production throughout the year, improvement of cultivation conditions to promote the synthesis of metabolites of interest, and ensure the production of these compounds in previously established quantities to obtain the desired biological effects, which allow the production of standardized extracts (ensure that each batch of extracts have the required levels of the compounds of interest). These could be made by improving the germination ratios, developing *in vitro* micropropagation techniques to improve plants multiplication rates, or for clonal propagation of selected plants (with higher contents of the bioactive compounds) to reduce batch variability. Micropropagation can also be used for faster production of stock plantlets to supply greenhouse planting (non-viable or low seed production, or low vegetative reproduction). Moreover, these could encourage the expansion of commercial applications of halophyte species from the Algarve as food (*e.g.* beverages or health supplements) cosmetics (*e.g.* anti-ageing or anti-melanogenic) or pharmaceutical ingredients. Further studies may also include the combination of various extracts from different halophytes intending to create a formulation with enhanced biological effects, as for example antioxidant, neuroprotective, antidiabetic or anti-hyperpigmentation. Moreover, the detected biological properties may be confirmed using different models, such as cellular-based and *in vivo* models.

6.3. References

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Nelson Mandela


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