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CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF HALOPHYTE PLANTS WITH ETHNOPHARMACOLOGICAL USE IN THE ALGARVE COAST

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Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo Ciências do Mar

Especialidade em Biotecnologia Marinha

Trabalho efetuado sob a orientação de:

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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.

(Catarina Guerreiro Pereira)

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.

"Caminante no hay camino", António Machado

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Lastly, to my companion, friend, lover and everything else that he is and means to me, I am deeply grateful to have you... and that you came along for the ride! To everything past, present and future and to our biggest adventure yet, Mariana!

Sê livre p’ra viver, não percas o tempo a chupar limão,
Naptel Xulima, tudo na vida rima se prestares atenção!

“Naptel Xulima”, HMB

ABSTRACT

This work aimed to investigate the potential of medicinal halophytes as sources of bioactive compounds with health-promoting properties, while validating their traditional uses and searching for new bioactivities/applications. Halophytes are salt-tolerant plants that survive in extreme biotopes and, to cope with environmental stress, are equipped with powerful defence mechanisms, including highly bioactive compounds. Several medicinal halophytes are used in folk therapeutics but, despite their ethnopharmacological importance, are still underexplored. This study focused on five medicinal halophytes from southern Portugal, namely *Artemisia campestris* L. subsp. *maritima* Arcangeli (dune wormwood), *Crithmum maritimum* L. (sea fennel), *Eryngium maritimum* L. (sea holly), *Helichrysum italicum* (Roth) G.Don subsp. *picardii* (Boiss&Reuter) Franco (everlasting), and *Plantago coronopus* L. (buckshorn plantain). Water and organic extracts were prepared from different plant organs, assessed for *in vitro* antioxidant, anti-diabetic, anti-hyperpigmentation and anti-protozoan activities, and chemically characterized.

The large majority of the extracts have high polyphenolic content and are a potentially good source of these bioactive phytochemicals. They presented a wide diversity of phenolics, especially coumaric, ferulic, syringic, chlorogenic, and *p*-hydroxybenzoic acids. Minerals were also analysed, and some species may have a nutritional role as mineral supplementary source, particularly sea fennel for macronutrients and dune wormwood for microelements. A preliminary toxicological assessment showed that extracts had overall low toxicity. As for bioactivities, results confirm the strong *in vitro* antioxidant capacity of the extracts. Everlasting, dune wormwood and sea holly also showed anti-diabetic activity, while dune wormwood had additional anti-hyperpigmentation capacity, and sea fennel had activity against *Trypanosoma cruzi*. In conclusion, all halophytes can be useful sources of antioxidants to potentially help prevent oxidative-stress related diseases, while everlasting, dune wormwood and sea holly may additionally help control glucose levels. Dune wormwood is also a prospective source of compounds to prevent skin darkening and sea fennel may provide effective anti-*T. cruzi* molecule(s).

Keywords: medicinal halophytes, phenolic compounds, minerals, antioxidant activity, anti-diabetic activity, anti-hyperpigmentation activity

RESUMO

Este trabalho teve como objetivo principal explorar o potencial de plantas halófilas medicinais enquanto fontes de compostos bioativos com aplicação terapêutica, e ao mesmo tempo validar os seus usos na "medicina" popular e procurar novas bioatividades e / ou aplicações. As plantas halófilas possuem uma elevada tolerância ao sal e completam o seu ciclo de vida em biótopos extremos, tais como zonas costeiras de influência salina. Para resistirem ao stress ambiental, estão equipadas com poderosos mecanismos de defesa que incluem a produção de compostos altamente bioativos, cujas atividades biológicas poderão ajudar a explicar a utilização de algumas destas plantas como remédios populares e para alimentação humana (e animal). De facto, inúmeras plantas halófilas são utilizadas no tratamento de várias doenças e infeções, principalmente em áreas rurais onde as plantas medicinais ainda são uma importante fonte terapêutica. No entanto, estas plantas são pouco exploradas e são também poucas as descrições das suas bioatividades, apesar da sua importância etnofarmacológica. Na região do Algarve (sul de Portugal), poucas espécies têm sido estudadas apesar do seu potencial conteúdo em moléculas bioativas. Assim, com o intuito de expandir o nosso conhecimento acerca das possíveis propriedades terapêuticas e promotoras de saúde e bem-estar deste tipo de plantas, este trabalho estudou cinco plantas halófilas medicinais comuns no Algarve, escolhidas pelos seus usos medicinais e potenciais atividades biológicas, nomeadamente *Artemisia campestris* L. subsp. *maritima* Arcangeli (madorneira), *Crithmum maritimum* L. (funcho marítimo), *Eryngium maritimum* L. (cardo marítimo), *Helichrysum italicum* (Roth) G.Don subsp. *picardii* (Boiss&Reuter) Franco (perpétua das areias) e *Plantago coronopus* L. (diabelha). O objetivo foi desvendar o seu potencial enquanto fontes de compostos e / ou extratos bioativos com aplicações terapêuticas, cosméticas e / ou nutricionais. Para tal, à semelhança de preparações tradicionais, foram preparados extratos aquosos e / ou orgânicos de diferentes órgãos (raízes, caules, folhas e / ou flores), e avaliados quanto às suas atividades antioxidante, antidiabética, anti-hiperpigmentação e anti-protozoária *in vitro.* Adicionalmente, para o funcho marítimo, cardo marítimo e perpétua das areias, os extratos foram estudados na sua dose de ingestão tradicional, i.e., usando a medida "chávena-de-chá" (e também a medida "gotas", no caso das tinturas do cardo), para analisar as plantas duma perspetiva de utilização medicinal típica. Os extratos foram ainda caracterizados quimicamente.

Os extratos foram caracterizados em relação aos compostos fitoquímicos presentes por métodos espectrofotométricos para determinar o seu conteúdo em fenólicos totais (TPC), para além do conteúdo em outros grupos fenólicos (flavonóides totais, taninos condensados, etc.).

Em geral, todos os extratos analisados têm um elevado conteúdo polifenólico e são, potencialmente, boas fontes destes compostos fitoquímicos bioativos. De um modo geral, os extratos da perpétua das areias tiveram o maior conteúdo em fenólicos totais, seguidos pelos extratos da madorneira, enquanto que o cardo marítimo teve o mais baixo TPC. De notar que, globalmente, os órgãos aéreos (folhas e flores) de todas as plantas mostraram ter conteúdos fenólicos mais elevados do que os restantes órgãos. Os extratos foram ainda caracterizados por Cromatografia Líquida de Alta Eficiência (HPLC) ou por Ultra-HPLC para determinar o seu perfil polifenólico e outros compostos tentativamente identificáveis. Foi encontrada uma grande diversidade de fenólicos nestas plantas halófilas, sendo os mais abundantes: verbascósido e luteolina-7-*O*-glucosídeo na diabelha, ácidos clorogénico, neo- e cripto-clorogénico no funcho marítimo, ácidos quínico e clorogénico na perpétua das areias e na madorneira, e carvacrol e naringenina no cardo marítimo. Os compostos fenólicos mais comuns foram os ácidos fenólicos, com uma ligeira prevalência dos ácidos hidroxicinâmicos. Os ácidos cumárico e ferúlico foram identificados nas cinco plantas, e os ácidos *p*-hidroxibenzoico, siríngico e clorogénico estavam presentes em pelo menos quatro das espécies. O conteúdo em minerais foi também analisado nos extratos das folhas da diabelha e dos órgãos do funcho marítimo, cardo marítimo e madorneira. O sódio foi o elemento mais abundante encontrado, e os outros minerais estavam presentes em valores representativos de uma pequena porção da ingestão diária recomendada para adultos. De um modo geral, os resultados salientaram um possível papel nutricional destas plantas enquanto fonte mineral suplementar, particularmente o funcho marítimo para os macronutrientes e a madorneira para os micronutrientes. Foi efetuada também uma avaliação toxicológica preliminar aos extratos do funcho marítimo, madorneira e perpétua das areias, através da sua capacidade para diminuir a viabilidade de diferentes linhas celulares. Os extratos apresentaram, de um modo geral, baixa toxicidade.

Os extratos foram também analisados quanto a propriedades antioxidante, antidiabética e anti-hiperpigmentação, através de uma bateria de ensaios para testar *in vitro* atividades de captação de radicais e de quelação de metais, capacidade de inibição de enzimas digestivas e de inibição da tirosinase, respetivamente. As decocções, tinturas e óleos essenciais da perpétua das areias e do funcho marítimo, foram também testados *in vitro* para a atividade anti-protozoária contra *Trypanosoma cruzi*, o agente causativo da doença de Chagas. Os extratos da perpétua das areias e da madorneira detiveram a maior capacidade antioxidante, seguidas pelos do funcho marítimo; contudo, os extratos do cardo marítimo demonstraram a mais potente atividade quelante do ferro. Os extratos da perpétua, madorneira e cardo revelaram ainda atividade antidiabética, apesar de apenas os do cardo serem capazes de inibir todas as enzimas

hidrolisantes de hidratos de carbono; contudo, os extratos da madorneira foram os mais eficientes a inibir a α-glucosidase, mais ainda que o controlo positivo (acarbose). Apenas os extratos da madorneira apresentaram capacidade de inibir a tirosinase. O extrato aquoso do funcho marítimo foi o mais ativo e seletivo contra *T. cruzi*. De um modo geral, os resultados confirmaram a forte capacidade antioxidante *in vitro* dos extratos, evidenciando que todas as cinco plantas halófilas podem ser úteis como fontes de moléculas ou produtos antioxidantes e, como tal, poderão ajudar a prevenir doenças relacionadas com stress oxidativo. O potencial antidiabético dos extratos da perpétua das areias, da madorneira e do cardo marítimo podem ainda auxiliar no controlo dos níveis de glucose, ajudando pacientes com *diabetes mellitus* tipo 2. Por outro lado, os extratos da madorneira são também possíveis fontes de compostos para prevenção / tratamento de hiperpigmentação da pele, enquanto que os do funcho marítimo poderão ser fontes de moléculas anti-*T. cruzi*.

No geral e em conclusão, o resultado deste trabalho demonstra que as cinco espécies de plantas halófilas estudadas são prospectivamente boas candidatas a serem utilizadas como alimento (as folhas da diabelha, por exemplo), em bebidas à base de plantas (como por exemplo tisanas de funcho e cardo marítimos e de perpétua das areias), como fontes de moléculas de relevo (ex.: compostos anti-*T. cruzi* do funcho marítimo), ou como matéria prima para as indústrias cosmética (como a madorneira para problemas de hiperpigmentação) e farmacêutica (ex.: a perpétua das areias, a madorneira e o cardo marítimo para o controlo da diabetes), e ainda para o segmento comercial de alimentos funcionais e / ou nutracêuticos (todas as cinco plantas halófilas enquanto antioxidantes potentes para prevenção de condições relacionadas com stress oxidativo). Adicionalmente, o perfil químico e atividades biológicas podem ajudar a explicar os usos tradicionais destas plantas.

Palavras Chave: plantas halófilas medicinais, compostos fenólicos, minerais, atividade antioxidante, atividade antidiabética, atividade anti-hiperpigmentação

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ABBREVIATIONS

AA	Amino acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	One-way analysis of variance
APCI	Atmospheric pressure chemical ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAE	Caffeic acid equivalents
CAT	Catalase
CCA	Copper chelating activity
CCE	Cyanidin chloride equivalents
CCV	Calibration verification standard
CD	Chagas Disease
CE	Catechin equivalents
CTC	Condensed tannin content
ddMS2	Data dependent fragmentation
DMACA	4-dimethylaminocinnamaldehyde
DMAPP	Dimethylallyl pyrophosphate
DPPH	1,1-diphenyl-2picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EO	Essential oils
EtOAc	Ethyl acetate
F-C	Folin-Ciocalteau
FRAP	Ferric reducing antioxidant power
FWHM	Full width at half maximum
GAE	Gallic acid equivalents
GHS	Reduced glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HAD	Hydroxycinnamic acid derivatives
HESI	Heated electrospray ionization
HepG2	Human hepatocellular carcinoma cells
Hex	Hexane
HPLC-DAD	High performance liquid chromatography – diode array detection
ICA	Iron chelating activity
IPP	Isopentenyl pyrophosphate
LC-HRMS	Liquid chromatography – high resolution mass spectrometry
LLC-MK2	*Macaca mulatta* kidney epithelial cells
LOQ	Limit of quantification
MeOH	Methanol
MEP	Methylerythritol pathway

MP-AES	Microwave Plasma-Atomic Emission Spectrometer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N9	Murine microglia cells
NF	Not found
NI	Not identified
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NTDs	Neglected tropical diseases
$OH^{\bullet}$	Hydroxyl radical
$O_2^{\bullet-}$	Superoxide radical
PG	Propyl gallate
QE	Quercetin equivalents
RE	Rutin equivalents
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RT	Retention times
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
U2OS	Human osteosarcoma cells
UHPLC-PDA-MS	Ultra-high performance liquid chromatography–photodiode array–mass-spectrometry
WHO	World Health Organization

OUTLINE OF THE THESIS

This thesis is divided in eight chapters. The first chapter corresponds to a general literature review of the main subjects of the thesis, chapters 2 to 7 describe the experimental work and obtained results in a publication format (research articles), and chapter 8 includes an overall discussion and conclusions of the main results along with future perspectives of this research. This thesis was designed to be based on research articles that have been published (chapters 2 to 5), are in press (chapter 6) or in preparation (chapter 7) for publication, and that provide an account of the research that supports this thesis. All presented publications were written to stand alone and, therefore, some repetition may be found in parts of the manuscript.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Halophyte Plants

Representing approximately 1% of the world's plant species, halophytes are salt-tolerant plants with particular morphological and physiological traits that make them well suited to survive and flourish in a multitude of saline biotopes. These environments, usually coastal ecosystems like beaches, rocky shores, estuaries, saltmarshes and lagoons, are exposed to highly fluctuating abiotic constraints namely salinity, light intensity, drought, temperature, among others (Grigore and Toma 2017; Hasanuzzaman et al. 2014; Ksouri et al. 2012). Halophytes are well adapted to these harsh conditions due to their high physiological plasticity comprised by various response mechanisms to handle abiotic stress, particularly salinity and drought, although species differ in the overall degree of stress tolerance. Most halophytes use selective ion accumulation and compartmentalization for internal osmotic adjustment, controlled ion uptake by roots and transport to leaves, synthesis and accumulation of compatible solutes to accommodate ionic balance, salt accumulation or secretion, and induction of antioxidant systems and other metabolites to maintain redox homeostasis; other adaptations may include succulence, salt glands, successive cambia or bulliform cells, found only in some halophytic species (Aslam et al. 2011; Flowers et al. 2010; Grigore and Toma 2017; Hasanuzzaman et al. 2014). The extreme environmental stress in which halophytes develop can particularly trigger the production and accumulation of reactive oxygen species (ROS) that, in turn, can lead to cellular and tissue damage, generating metabolic disorders and senescence processes. To cope with these excessive ROS, halophyte plants are equipped with powerful antioxidant defence systems, including antioxidant enzymatic mechanisms and enhanced synthesis of antioxidant secondary metabolites (phenolics, terpenoids, vitamins and other molecules) that counteract ROS production, inhibit oxidative chain-reactions and protect cellular structures from oxidative effects (Ksouri et al. 2008, 2012). Apart from antioxidant activity (e.g. radical-scavenging and redox metal-chelating), most of these metabolites display additional biological activities such as enzyme inhibition, antimicrobial, anti-inflammatory, antitumoral, among others, leading to beneficial therapeutic properties that help explain the use of some halophytes in traditional medicine and as dietary plants (Ksouri et al. 2012; Shahidi and Ambigaipalan 2015).

Halophyte plants have been studied from several different scientific perspectives (ecology, botany, plant physiology, biochemistry, molecular biology, biotechnology, etc.) rendering a plethora of classifications to define them based on different criteria such as salt requirements, adaptive mechanisms or biotope characteristics. Until today, due to their

taxonomical and ecological complexity, no consensus has been reached and it remains a matter of much debate (Grigore and Toma 2017; Hasanuzzaman et al. 2014; Ksouri et al. 2012). From an ecological perspective, it is logical to associate halophytes to the saline ecosystems they live in. Hence, plants that grow in saline environments can be considered halophytes given their close dependence on soil salinity, despite its varying degrees (Grigore and Toma 2017). In this sense, halophytes can be classified as obligate, facultative, or habitat-indifferent. Obligate halophytes (also called true halophytes) only grow in salty habitats, requiring saline conditions for optimum growth and development (e.g. members of the Amaranthaceae family). Facultative halophytes tolerate salt and grow in saline soils, although their optimum is in salt-free or low-salt conditions (e.g. members of Poaceae, Cyperaceae, and Juncaceae families). Habitat-indifferent plants (or supportive halophytes) are able to cope with salt, but usually grow on salt-free conditions (e.g. some species of the Poaceae, Juncaceae, and Amaranthaceae families) (Aslam et al. 2011; Hasanuzzaman et al. 2014). Nevertheless, this classification is still controversial as some authors defend that only true halophytes should be considered halophyte plants due to the mandatory salt requirements, while others believe that facultative and supportive halophytes deserve their place in the halophyte category since they occupy niches in saline ecosystems (Grigore and Toma 2017). Usually, the parameter to separate a halophyte from a non-halophyte (glycophyte, a non-salt tolerating plant species, 'sensitive' to saline conditions) has been the tolerance to salt (NaCl) concentration, with a dividing line established at 0.5% NaCl (~86 mM). Additionally, a limit of 200 mM NaCl tolerance has also been suggested to separate true halophytes from salt-tolerant plants (Flowers et al. 2010; Santos et al. 2015). However, these limits can be quite reductive when considering the very broad spectrum of salt tolerance, from the least (e.g. chickpea dies in 25 mM NaCl) to the most salt-tolerant species (e.g. *Arthrocnemum macrostachyum* (Moric.) Moris and *Tecticornia* spp. survive in up to 1000 mM NaCl) (Flowers et al. 2010; Grigore and Toma 2017; Hasanuzzaman et al. 2014; Santos et al. 2015).

More than 2500 plant species with some level of salinity tolerance are known worldwide; they are distributed through a large number of plant families and have a wide ecological spectrum (Flowers et al. 2010; Grigore and Toma 2017; Ksouri et al. 2012). Perhaps the most promising value of halophytes is their potential to grow and be produced in otherwise uncultivable saline soils and marine-influenced environments, being irrigated with salt water. Most of our crop species are 'salt-sensitive' glycophytes whose cultivable areas are diminishing due to soil salinization and desertification. In the present context of increasing world population and global climate change, this threat to agriculture and food production could be overcome

with alternative resources such as halophytes. In fact, several halophyte species can be suitable candidates to serve as alternative cash crops to be explored in diverse commercial segments, from human and animal nutrition to pharmaceutical and cosmetic industries, serving as an approach for the sustained management of saline areas, for revegetation, soil-remediation or even 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). In the scientific environment, halophyte plants are currently considered and increasingly proved to be an important promising (and almost untapped) reservoir of bioactive molecules with multiple possible biotechnological applications (Barreira et al. 2017; Custódio et al. 2012; Ksouri et al. 2012; Lopes et al. 2016; Oliveira et al. 2016, 2018; Pereira et al. 2017a,b,c, 2018; Petropoulos et al. 2018a,b; Rocha et al. 2017; Rodrigues et al. 2014, 2015, 2016, 2017a,b,c, 2018a,b). Moreover, many halophytes have been and are still traditionally used all over the world as food and folk remedies, which stands to prove their great potential still largely unexplored (Ksouri et al. 2012).

1.1.1. Ethnopharmacological uses of halophytes

Some halophytes are edible and consumed as vegetables or salad ingredients (e.g. *Crithmum maritimum* L., *Daucus carota* L., *Eryngium maritimum* L., *Portulaca oleracea* L., *Atriplex*, *Chenopodium*, *Plantago*, *Polygonum*, *Salicornia*, *Salsola* and *Sarcocornia* species, etc.) in countries such as Italy, France, Syria and in the Balcans (Al-Oudat and Qadir 2011; Guarrera and Savo 2016; Ksouri et al. 2012; Redzic 2006), or used for animal feeding (e.g. *Arthrocnemum*, *Atriplex* and *Tamarix* species) (Al-Oudat and Qadir 2011; Ksouri et al. 2012; El Shaer 2006). But more than being a food resource, several halophytes are used as traditional remedies to treat various diseases and infections particularly in rural areas where folk medicine remains a major therapeutic source. Yet, for most of those plants there is no scientific evidence supporting their therapeutic claim (Ksouri et al. 2012). Researchers have long been compiling and categorizing the ethnomedicinal uses of plants in different areas of the world, namely the Mediterranean region (González-Tejero et al. 2008), in Italy (Cornara et al. 2009), Portugal (Neves et al. 2009; Novais et al. 2004), Egypt (AbouZid and Mohamed 2011), or the North Sea (Liebezeit et al. 1999), but studies dedicated specifically to the ethnobotany of halophytes are uncommon (Al-Oudat and Qadir 2011; Ksouri et al. 2008, 2012; Qasim et al. 2011).

Ksouri et al. (2012) is, in the author's opinion, the best comprehensive review committed to detail exclusively the folkloric medicinal uses of halophyte plants, grouped by plant families,

discussing their claimed beneficial health properties along with reported biological activities and phytoconstituents. Those authors describe up to 20 plant families (Aizoaceae, Apiaceae, Asteraceae, Brassicaceae, Amaranthaceae, Combretaceae, Elaeagnaceae, Fabaceae, Lamiaceae, Liliaceae, Malvaceae, Moraceae, Palmae, Plantaginaceae, Plumbaginaceae, Poaceae, Rubiaceae, Tamaricaceae, Verbenaceae, and Zygophyllaceae) containing more than 70 species of medicinal halophytes used in the treatment of various human ailments. Qasim et al. (2011), in its compilation of coastal halophytic medicinal species along the Arabian Sea, reports 21 plant families (14 of which not mentioned by Ksouri et al. [2012]: Asclepiadaceae, Boraginaceae, Capparaceae, Convolvulaceae, Cucurbitaceae, Cyperaceae, Euphorbiaceae, Mimosaceae, Portulacaceae, Rhamnaceae, Rhizophoraceae, Salvadoraceae, Solanaceae, and Tiliaceae), with a total of 45 halophyte species with folk uses (13 of them recorded in Ksouri et al. [2012]). Al-Oudat and Qadir (2011) present an extensive list of Syria's halophytic flora with more than 90 halophytes in 27 plant families having traditional uses (medicinal, food or fodder); 9 plant families (Amaryllidaceae, Caryopphyllaceae, Cistaceae, Frankeniaceae, Juncaceae, Papilionaceae, Polygonaceae, Thymelaeaceae, and Typhaceae) and around 80 halophytes are not mentioned by Ksouri et al. (2012) nor Qasim et al. (2011). These three studies show the high diversity of halophytes used by local populations, attesting to the importance of this group of plants in folk medicine. The halophytes described are used to treat almost all types of conditions being the most common uses antimicrobial and antiparasitic, analgesic, antipyretic, anti-inflammatory, diuretic, to treat digestive and respiratory disorders, skin conditions (dermatitis, wounds, burns) or specific diseases (tuberculosis, scurvy, diabetes, cancer). When applied as therapeutics, the whole plant or plant organs (usually roots, leaves or flowers), fresh or dried, are typically eaten raw or cooked, applied as cataplasm (poultice), or more commonly ingested as infusions, decoctions, or alcoholic extracts (tinctures) (Al-Oudat and Qadir 2011; Ksouri et al. 2012; Qasim et al. 2011).

While the purpose of this work is not to compile a review of medicinal halophyte species recorded in literature, examples of halophytic folk remedies can be given in a general overview. In this sense, Table 1.1 is a compilation of halophyte plants and their traditional medicinal uses mostly in the Mediterranean region, found in some studies besides the ones mentioned above (Al-Oudat and Qadir 2011; Ksouri et al. 2012; Qasim et al. 2011). The eHALOPH database was used to identify halophyte species (eHALOPH 2018). Briefly, in the Mediterranean region, halophytes such as *Ammi visnaga* (L.) Lam., *Centaurium spicatum* (L.) Fritsch., *Cynodon dactylon* (L.) Pers., *Dittrichia viscosa* (L.) Greuter, *E. maritimum*, *Plantago coronopus* L., *Polygonum aviculare* L., or *Spergularia rubra* (L.) J.&C.Presl. are some of the plants

comprised in the populations' collective knowledge to treat conditions related to several pathologies like respiratory, digestive or skin disorders, among others (González-Tejero et al. 2008). In Italy, *Asparagus officinalis* L. and *Lavatera arborea* L. are used as diuretic, *C. maritimum* and *Helichrysum italicum* (Roth) G.Don as anti-inflammatory, and *D. carota* as laxative (Cornara et al. 2009). In Tunisia, *Mesembryanthemum edule* L. (syn. *Carpobrotus edulis* L.) and *M. crystallinum* L. are used for their antiseptic and antimicrobial properties (Ksouri et al. 2008); *M. edule* is also commonly used in South Africa for oropharyngeal infections and to treat wounds, stings and burns (Falleh et al. 2011; Springfield et al. 2003). Still in Tunisia, *Limoniastrum monopetalum* (L.) Boiss. is used against infectious diseases or parasites (Trabelsi et al. 2010) and *Tamarix gallica* L. is used as diuretic and laxative (Ksouri et al. 2008). In Egypt, *P. olearacea* is used for rheumatic pain (AbouZid and Mohamed 2011) while it also has recorded uses in Albania and Cyprus for cardiovascular and muscular conditions (González-Tejero et al. 2008). But perhaps the medicinal halophyte with most uses and in the higher number of Mediterranean countries, as well as in Iraq and Iran, is *Pistacia lentiscus* L.; from its numerous applications the more common ones are analgesic, antipyretic, anti-inflammatory, for skin and gastrointestinal disorders (Aflosul 2006; Ali-Shtayeh et al. 1998; Bozorgi et al. 2013; González-Tejero et al. 2008; Novais et al. 2004). In other areas, like the North Sea region for example, some halophyte species with traditional uses are *Artemisia maritima* L., as anthelmintic, *Limonium vulgare* Mill. and *Salsola kali* L., both anti-diarrheic (Liebezeit et al. 1999). *Artemisia maritima* is also a diabetes treatment in North East India (Sheikh et al. 2015) and *S. kali* is known as hypotensive in Tunisia (Ksouri et al. 2008).

In Portugal, ethnobotanical surveys have referenced some halophytes as plants with medicinal uses (Table 1.1). *Achillea millefolium* L. and *Raphanus raphanistrum* L. are used as antipyretic, while *Arundo donax* L., *D. carota*, *Eucalyptus camaldulensis* Dehnh. and *P. coronopus* help treat colds and cough. Plants with described anti-inflammatory properties are *A. millefolium* and *Plantago* species; *Plantago* species are also reported as analgesic along with *D. carota* and *P. lentiscus*. The latter species is also employed to address rheumatic issues, just like *C. dactylon* and *Artemisia campestris* L. subsp. *maritima* Arcangeli. To handle digestive disorders, including diarrhoea, *A. campestris* subsp. *maritima*, *Chenopodium ambrosioides* L., *C. dactylon*, *D. carota*, *Plantago major* L., *P. lentiscus*, *P. aviculare* and *Rumex crispus* L. are described, and some of these plants (*C. dactylon*, *C. ambrosioides*, *P. lentiscus* and *P. aviculare*), along with *A. millefolium*, are also used as diuretic. For skin infections, wounds or with emollient properties, *Chenopodium album* L., *E. camaldulensis*, *H. italicum*, *P. aviculare*, *P. lentiscus*, *Typha dominguensis* Pers., *Mesembryanthemum* and *Plantago* species are the

species reported. *Helichrysum italicum* and *A. campestris* subsp. *maritima* also have anthelmintic uses. Additionally, *Cotula coronopifolia* L. is used for bladder issues, *D. viscosa* for sciatic pain, *Salicornia ramosissima* J.Woods and *Sarcocornia fruticosa* (L.) A.J.Scott to address obesity and *Tamarix africana* Poir. for tooth ache and abscesses (Aflosul 2006; Almargem 2018; Bozorgi et al. 2013; Carapeto 2006; Carvalho 2006; Neves et al. 2009; Novais et al. 2004; Viegas et al. 2014).

The length of Table 1.1, which aimed at giving only some examples of medicinal halophytes found in few studies, demonstrates the importance and extensive uses of halophyte plants in folk medicine, particularly in the Mediterranean region. Herbal medicine has always had a fundamental role in human welfare and it is still of utmost importance in many cultures today. In this sense, the ethnopharmacological knowledge of populations can serve as base to search for novel bioactive compounds. Considering this, scientific research has explored and produced numerous studies about medicinal plants' biological properties and chemical constituents not only to advance plant drug discovery but also to validate the plant's traditional uses. However, for halophytes with folk uses, research on their bioactivities and subsequent evidence supporting their medicinal claim is still scarce for many of those plants.

Table 1.1 Examples of medicinal halophytes used in different countries / regions of the world and their therapeutic applications.

Plant Family / species	Medicinal use	Plant organs and / or administration	Country / region	Ref.*
Aizoaceae				
Mesembryanthemum crystallinum L.	As antiseptic	–	Tunisia	[3]
Mesembryanthemum edule L. (synonym *Carpobrotus edulis* L.)	To treat microbial infections, sinusitis, diarrhoea, eczema, tuberculosis, and as antiseptic; for oropharyngeal infections and to treat stings wounds and burns; to treat fungal skin infections	Leaves, sap	Tunisia South Africa Portugal	[3] [4][5] [15]
Mesembryanthemum nodiflorum L.	For alopecia, skin infections, burns and wounds	Aerial parts infusions, sap	Portugal	[15]
Amaranthaceae				
Atriplex leucoclada Boiss.	To treat cough and sore throat, emollient	Leaves	Iran	[12]
Anabasis articulata (Forssk.) Moq.	To treat skin conditions	–	Egypt	[1]
Beta vulgaris L.	For digestive ailments	–	Cyprus	[1]
Chenopodium album L.	To treat wounds	Aerial parts and sap	Portugal	[15]
Chenopodium ambrosioides L.	To treat digestive disorders, as diuretic	Aerial parts infusions and decoctions	Portugal	[15]
Salicornia ramosissima J.Woods	To address obesity	Fresh branches and leaves	Portugal	[16]
Sarcocornia fruticosa (L.) A.J.Scott	To address obesity	Fresh branches and leaves	Portugal	[16]
Salsola kali L.	For cardiovascular problems; hypotensive; anti-diarrhoea	–	Cyprus; Tunisia North Sea	[1][3] [10]
Salsola soda L.	For cardiovascular problems	–	Cyprus	[1]
Anacardiaceae				
Pistacia lentiscus L.	For digestive, skin and sensory ailments; anti-inflammatory, antipyretic, astringent, to treat diarrhoea, throat infections, renal stones; appetizer, diuretic, anti-rheumatic, analgesic, antiseptic, emollient, to treat diabetes, eczema,	Roots, aerial parts, seeds, bark, resin, infusions, alcoholic and vinegar macerations and poultices	Algeria, Cyprus, Morocco Palestine Algeria, Greece, Iraq, Iran, Italy, Jordan, Spain, Portugal, Tunisia, Turkey	[1] [8] [9][13] [15]

	hypertension, influenza, diarrhoea, asthma, infections, paralysis, allergies, etc.			
Apiaceae				
Ammi visnaga (L.) Lam.	Kidney, respiratory and cardiovascular conditions	–	Algeria, Egypt	[1][7]
Crithmum maritimum L.	To treat urinary and prostate inflammations, colics	Aerial organs decoctions	Italy	[2]
Daucus carota L.	As laxative; analgesic, to treat gastroenteritis and diarrhoea, cough, colds and kidney stones	Leaves and root infusions and syrup	Italy; Portugal	[2] [15][17]
Eryngium maritimum L.	For respiratory pathologies	–	Cyprus	[1]
Asclepiadaceae				
Calotropis procera (Ait.) Ait.	To treat respiratory and digestive disorders	–	Egypt	[1]
Asparagaceae				
Asparagus officinalis L.	As diuretic	Shoot's decoction	Italy	[2]
Asteraceae				
Achillea millefolium L.	For skin conditions; antipyretic, diuretic, anti-inflammatory	Rhizome and aerial parts infusions	Italy Portugal	[1] [14]
Artemisia campestris L. ssp *maritima* Arcangeli	To treat gastric disorders, hypertension and rheumatic, anthelmintic and abortifacient	Stems and leaves infusions and decoctions	Portugal	[19]
Artemisia maritima L.	Anthelmintic, insecticide; anti-diabetic	–	North Sea; India	[10][11]
Artemisia scoparia Waldst.&Kitam.	To treat indigestion and sore throat, emollient	Inflorescence	Iran	[12]
Cotula coronopifolia L.	To treat bladder issues	Flowers infusion	Portugal	[16]
Dittrichia viscosa (L.) Greuter	Muscular-skeletal, cardiovascular, digestive and skin illnesses; haemostatic and wound healing; for sciatic pain	Leaves Bathe in boiled roots	Algeria, Cyprus, Spain, Italy Portugal	[1] [2] [15]
Helichrysum italicum (Roth) G.Don	To treat sleeplessness, digestive disorders, cough, colds, asthma, allergies, and herpes, as analgesic, anti-inflammatory and anthelmintic; for dermatological conditions, including wounds	Leaves and flowers infusions and decoctions, essential oils	Italy, Spain Portugal	[2][18]
Brassicaceae				

Anastatica hierochuntica L.	To treat reproductive problems	–	Egypt	[1]
Raphanus raphanistrum L.	To treat sunstroke (antipyretic), colds and diabetes	Aerial parts boiled	Portugal	[15][16]
Caryophyllaceae				
Spergularia rubra (L.) J.&C.Presl.	For kidney diseases	–	Morocco, Spain	[1]
Cyperaceae				
Cyperus rotundus L.	Digestive, reproductive, muscular-skeletal and kidney disorders	–	Egypt	[1]
Scirpus holoschoenus L.	(not specified)	–	Spain	[1]
Elaeagnaceae				
Elaeagnus angustifolia L.	Anti-diarrhoea, hepatoprotective and analgesic	Fruits	Iran	[12]
Fabaceae				
Acacia nilotica (L.) Willd. ex Delile	To treat muscular-skeletal and sensory conditions	–	Egypt	[1]
Prosopis farcta (Banks & Sol) Macbr	Anti-diabetic	Fruits	Iran	[12]
Gentianaceae				
Centaurium spicatum (L.) Fritsch.	Cardiovascular, kidney, respiratory, muscular-skeletal and mental-nervous issues	–	Egypt, Italy	[1]
Malvaceae				
Lavatera arborea L.	Used as digestive, diuretic, against cystitis, cough and pain	Aerial organs poultices, infusions, decoctions	Italy	[2]
Myrtaceae				
Eucalyptus camaldulensis Dehnh.	Respiratory, skin and muscular-skeletal disorders; to treat wounds, asthma and colds	Boiling leaves' vapour	Cyprus, Italy Portugal	[1] [17]
Palmae				
Hyphaene thebaica (L.) Mart.	For hypertension and indigestion	Fruits decoction	Egypt	[7]
Plantaginaceae				
Plantago coronopus L.	To treat kidney and reproductive problems; analgesic, emollient, to treat cough and laryngitis	Aerial parts infusions	Cyprus Portugal	[1][13] [14][15]

		and decoctions		[17]
Plantago lanceolata L.	For respiratory and skin conditions; antiseptic; analgesic, antiseptic, mucolytic, anti-inflammatory, emollient	Leaves and flowers infusions	Algeria, Cyprus, Spain Italy; Portugal	[1] [2][14]
Plantago major L.	Skin, kidney, digestive, respiratory, muscular-skeletal and mental-nervous issues; wound healing, anti-haemorrhagic, anti-inflammatory, diuretic, gastroprotector	Leaves infusions and decoctions	Albania, Italy, Spain Portugal	[1] [13][14][15]
Poaceae				
Arundo donax L.	To treat infections, colds and swelling	Roots	Portugal	[15][16]
Cynodon dactylon (L.) Pers.	Kidney and digestive pathologies; diuretic, antipyretic; gastroprotector, to treat rheumatic and hepatitis	Roots decoctions and syrup	Cyprus Italy; Portugal	[1] [2][14][15]
Polygonaceae				
Polygonum aviculare L.	For digestive and skin problems; anti-haemorrhagic, sedative, diuretic, laxative, antiseptic, to treat wounds and stomach ulcers	Whole plant, infusions and decoctions	Cyprus, Italy Portugal	[1] [14][15][17]
Rumex crispus L.	Anti-diarrhoea	Seeds infusions	Portugal	[13]
Portulacaceae				
Portulaca oleracea L.	Cardiovascular, muscular-skeletal and mental-nervous disorders; antiparasitic; for rheumatic pain, as appetizer	Roots, stems, leaves Boiling leaves' vapour	Albania, Cyprus Iran Egypt	[1] [12] [7]
Plumbaginaceae				
Limoniastrum monopetalum (L.) Boiss.	To treat infectious diseases or parasites that cause painful and bloody diarrhoea	Leaves and galls infusions	Tunisia	[6]
Limonium vulgare Mill.	Anti-diarrhoea	–	North Sea	[10]
Salvadoraceae				
Salvadora persica L.	To treat digestive conditions	–	Egypt	[1]
Tamaricaceae				

Tamarix africana Poir.	For tooth ache and abscesses	Inner bark, boiled	Portugal	[15]
Tamarix gallica L.	Used as diuretic, expectorant, laxative, astringent	Aerial parts	Tunisia	[3]
Tamarix ramosissima Ledeb.	Wound healing	Leaves and resin	Iran	[12]
Typhaceae				
Typha domingensis Pers.	Antipyretic; to treat burns	Rhizome, pollen, leaves	Iran; Portugal	[12][15]
Zygophyllaceae				
Fagonia mollis Delile	To treat skin pathologies	–	Egypt	[1]
Peganum harmala L.	Cardiovascular, digestive, mental-nervous issues; antiseptic, analgesic and soporific	Fruits and seed	Algeria, Egypt Iran	[1] [12]
Zygophyllum coccineum L.	Respiratory, cardiovascular, digestive, skin, kidney and muscular-skeletal problems	–	Egypt	[1]

*References: [1] González-Tejero et al. 2008; [2] Cornara et al. 2009; [3] Ksouri et al. 2008; [4] Springfield et al. 2003; [5] Falleh et al. 2011; [6] Trabelsi et al. 2010; [7] AbouZid and Mohamed 2011; [8] Ali-Shtayeh et al. 1998; [9] Bozorgi et al. 2013; [10] Liebezeit et al. 1999; [11] Sheikh et al. 2015; [12] Ghasemi et al. 2013; [13] Novais et al. 2004; [14] Neves et al. 2009; [15] Aflosul 2006; [16] Carapeto 2006; [17] Carvalho 2006; [18] Viegas et al. 2014; [19] Almargem 2018.

1.2. Biological Activities of Halophytes

Several examples of claimed medicinal uses of a plant have been successfully related to a biological activity, leading to the development of valuable plant-derived drugs (e.g. atropine, found in *Atropa belladonna* L.; digoxin, from *Digitalis lanata* Ehrh.; the opiate morphine, from *Papaver somniferum* L.; paclitaxel, found in *Taxus brevifolia* Nutt.; tubocurarine, from *Chondrodendron tomentosum* Ruiz&Pav. bark, etc.) (Stepp and Thomas 2005). A biological activity can be defined as the inherent ability of a substance (regarding its concentration) to produce an effect in a biological process. Measuring said biological activities, usually through specific bioassays that assess the potency of an agent for the aimed biological reaction, allows the discovery and development of new biologically active compounds (Jackson et al. 2007). New compounds, along with the source of those compounds, can become important products or material for the pharmaceutical, cosmetic and / or food industries.

Contemporary research exploring the biochemical properties of medicinal plants reports biological activities in some halophytes, frequently validating their ethnomedicinal use. Again, Ksouri et al. (2012) gives a comprehensive review of bioactivities found in different medicinal halophyte species, namely antioxidant activity in *Atriplex halimus* L. or *M. edule*, cytotoxic effects of *Plantago* species, anti-inflammatory potential of *P. lanceolata* and *P. major*, antibacterial properties in *Cynara cardunculus* L. or *T. gallica*, antifungal activity in *Cakile maritima* Scop., neuroprotective potential of *Thespesia populnea* (L.) Sol. ex Corrêa, or hypoglycaemic / anti-diabetic properties in *Suaeda fruticosa* Forssk. ex J.F.Gmel. and *Capparis decidua* (Forssk.) Edgew., activities associated to biologically active molecules found on those species (like phenolic compounds, for example). Other authors, particularly in 'post-Ksouri review' studies, also found antioxidant potential in many halophytes (Amessis-Ouchemoukh et al. 2014; Jallali et al. 2014; Oueslati et al. 2012; Qasim et al. 2016; Petropoulos et al. 2018a,b; Stanković et al. 2015; Zengin et al. 2018), along with antimicrobial effects in *C. maritimum*, *Inula crithmoides* L. or *Limoniastrum* species (Jallali et al. 2014; Trabelsi et al. 2013), anti-inflammatory properties in *S. fruticosa*, *C. dactylon* or *T. gallica* (Oueslati et al. 2012; Petropoulos et al. 2018a), cytotoxic activity in *S. fruticosa*, *A. halimus* or *Beta vulgaris* L. (Oueslati et al. 2012; Petropoulos et al. 2018a,b), anti-cholinesterase / neuroprotective potential in *E. maritimum* and *P. lentiscus* (Amessis-Ouchemoukh et al. 2014) or even anti-diabetic properties in *A. macrostachyum*, *Halimione portulacoides* (L.) Aellen or *Salicornia europaea* L. (Zengin et al. 2018). However, it is noteworthy that reports of biological activities in halophytes from the Portuguese coast have only recently started to be unveiled. Figure 1.1

shows examples of halophyte species found in the Algarve (Portugal) coast, featured in such reports. Neuroprotective properties have been found in *A. macrostachyum*, *C. edulis* (Custódio et al. 2012), *Juncus acutus* L. (Rodrigues et al. 2017a), *Polygonum maritimum* L. (Rodrigues et al. 2018a) and *Armeria pungens* (Link) Hoffmanns&Link (Rodrigues et al. 2018b), while anti-inflammatory potential was detected in *P. coronopus* (Rodrigues et al. 2014), *Limonium algarvense* Erben (Rodrigues et al. 2016) and *P. maritimum* (Rodrigues et al. 2017b) (species in Fig. 1.1). *P. maritimum* has also shown anti-diabetic effects (Rodrigues et al. 2017b), whereas *J. acutus* also has cytotoxic potential (Rodrigues et al. 2014). Moreover, anti-melanogenic activities were present in *P. lentiscus* (Lopes et al. 2016) and *A. pungens* (Rodrigues et al. 2018b) (species in Fig. 1.1). Additionally, some halophytes from the Algarve (Portugal) coast have also shown to be potential source of molecules against neglected tropical diseases, namely *J. acutus* against *Trypanosoma cruzi* (Oliveira et al. 2016) and *I. crithmoides* and *S. rubra* against *Leishmania infantum* (Oliveira et al. 2018) (species in Fig. 1.1). These studies also report the antioxidant potential present in most of those halophytic species. In fact, from the array of bioactivities that can be found in halophyte species, antioxidant activity attracts the greater interest.

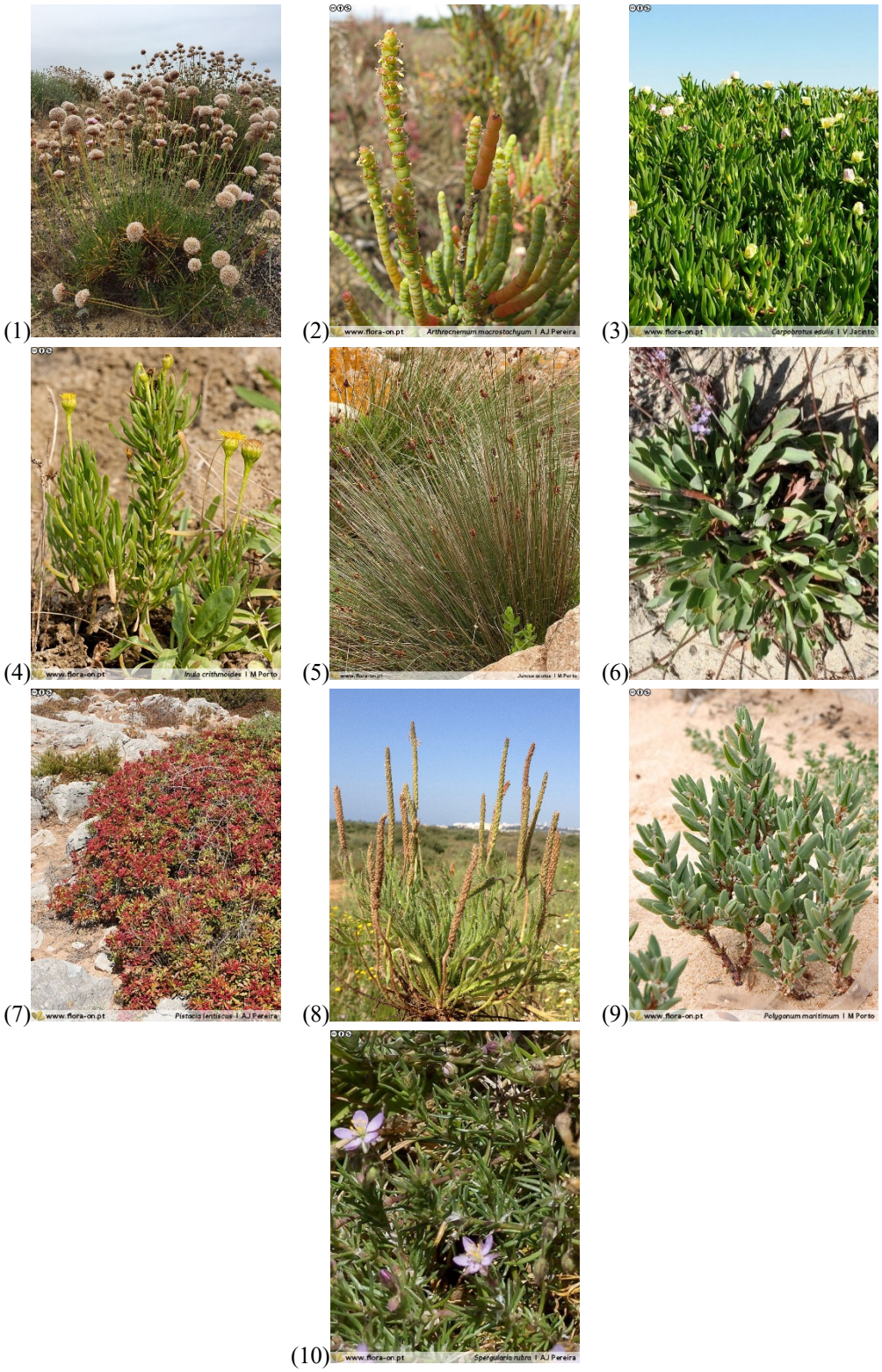

Figure 1.1 Some halophyte species from the Portuguese coast: (1) *Armeria pungens*, (2) *Arthrocnemum macrostachyum*, (3) *Carpobrotus edulis* (syn. *Mesembryanthemum edule*), (4) *Inula crithmoides*, (5) *Juncus acutus*, (6) *Limonium algarvense*, (7) *Pistacia lentiscus*, (8) *Plantago coronopus*, (9) *Polygonum maritimum*, (10) *Spergularia rubra*. Photos by the author (1 and 8), http://flora-on.pt (2 – 5, 7, 9,10) and http://www.virboga.de (6).

1.2.1. Antioxidant properties

Reactive oxygen species (ROS) are naturally formed in metabolic processes, such as the respiratory electron transport chain, due to the capacity of the oxygen molecule to accept an additional electron, producing the oxygen free radical, superoxide anion ($O_2^{\bullet-}$). The majority of superoxide produced comes from the mitochondrial respiratory chain; ubisemiquinone species generated in the electron transport reactions donate electrons to oxygen, providing a constant source of superoxide. To protect against the toxicity of this free radical, the antioxidant enzyme, superoxide dismutase (SOD) dismutates superoxide anion producing the non-radical, hydrogen peroxide (H_2O_2). Superoxide and hydrogen peroxide are the primary ROS derived from mitochondria, but in the presence of ferrous iron, and other redox metals such as copper, hydrogen peroxide can participate in the Fenton reaction producing the highly reactive and toxic hydroxyl radical ($OH^{\bullet}$).

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Reactive nitrogen species (RNS) are also produced in cellular redox processes; they include nitric oxide (NO), which participates in a wide range of cellular processes, as for example, as signalling molecule in neurons and in the immune system. ROS / RNS perform some important roles, acting as signalling molecules or as chemical defence against microorganisms, but they can also cause damage to structure and function of biomolecules. In addition to the endogenous sources, exogenous factors such as UV and ionizing radiation, exposure to pollutants, drugs and stress can also generate ROS / RNS (Figure 1.2) (Pisoschi and Pop 2015; Raha and Robinson 2000; Rashid et al. 2013; Saeidnia and Abdollahi 2013; Sen and Chakraborty 2011). In living systems, a balance is maintained between the production of reactive species and their neutralization by antioxidant defences, a redox balance, but overproduction of the first and / or diminishing of the second leads to an impairment in that redox balance (Figure 1.3) (Rashid et al. 2013; Sen and Chakraborty 2011). An enhanced level of ROS / RNS has deleterious effects as they can target almost all molecules in the cell. Lipids are the most susceptible to undergo oxidation, which is highly damaging to cell membranes, but ROS / RNS can also oxidise both the backbone and side chain of proteins, resulting in activity loss, and damage nucleic acids, leading to DNA mutations. Damage to cellular macromolecules and deregulation of cellular functions can, ultimately, lead to cell death, altered signalling pathways and degenerative processes (Figure 1.3). This imbalance between ROS / RNS and the organism's capacity to counteract them and subsequent potential damage is called oxidative / nitrosative stress and its severity is associated not only with aging but also with various diseases as, for example,

neurodegeneration, inflammation, carcinogenesis, diabetes and cardiovascular disorders. (Figure 1.3) (Pisoschi and Pop 2015; Rashid et al. 2013; Sen and Chakraborty 2011).

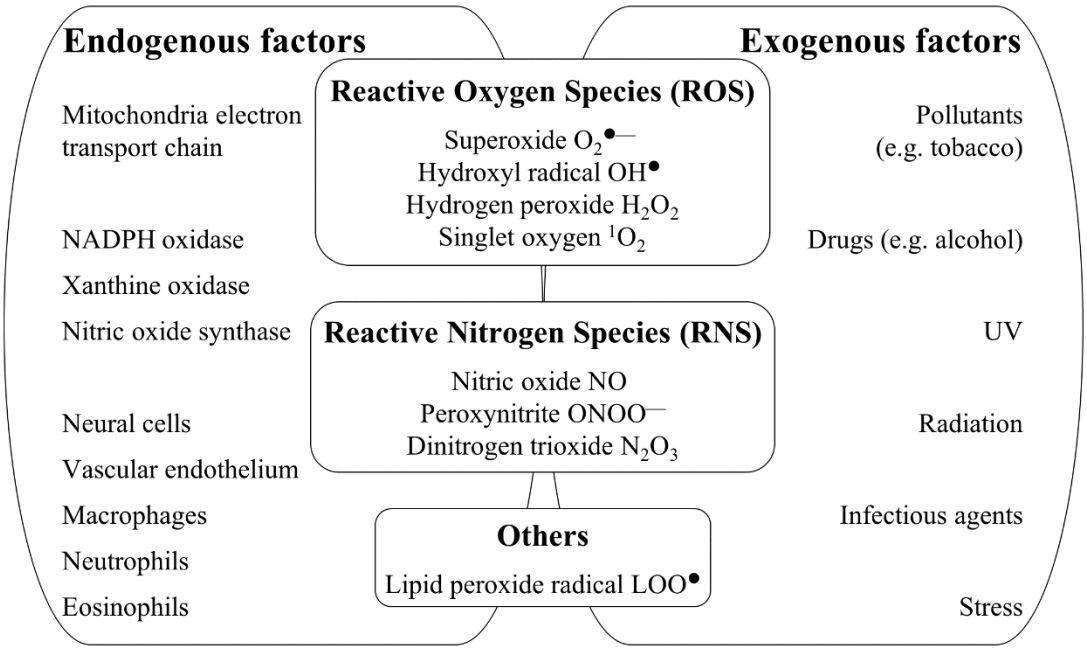

Figure 1.2 Examples of ROS / RNS and of their sources from endogenous and exogenous factors (adapted from Thanan et al. 2015).

To counteract oxidative stress, organisms have complex antioxidant defence systems that include antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx], and glutathione reductase [GR]) and non-enzymatic antioxidants, namely metabolic (e.g.: reduced glutathione [GHS], coenzyme Q_{10}, uric acid, metal-chelating proteins) and nutrient / dietary (e.g.: vitamins E and C, carotenoids, flavonoids, omega-3 and -6) molecules (Figure 1.3) (Rashid et al. 2013; Saeidnia and Abdollahi 2013; Sen and Chakraborty 2011). Antioxidants exert their protective role by reducing the production of ROS / RNS, scavenging, stabilizing, deactivating, and / or destroying ROS / RNS, or binding to or inactivating metal ions that generate ROS. They are crucial in delaying or inhibiting oxidative stress and its cell damaging effects and, therefore, in maintaining ideal cellular and systemic conditions (Saeidnia and Abdollahi 2013; Sen and Chakraborty 2011). Considering the vital role of antioxidants to living organisms, their use is one approach to prevent or reduce the severity of diseases in which oxidative stress is an underlying factor, being recognized as important components in maintaining optimum health and wellbeing. Additionally, they can function as immune modulators and be used as prophylaxis or adjuvant therapy (Sen and Chakraborty 2011). In this sense, studies about natural and powerful antioxidants have gained

much momentum in the pursuit of antioxidant intake to boost the endogenous antioxidant system, either as part of a wholesome antioxidant diet or as nutritional supplements. Moreover, antioxidants are fundamental food additives that prevent oxidation and food spoilage and help maintain nutritional and organoleptic properties. They are also routinely used in the cosmetic and pharmaceutical industries to protect against oxidation particularly in products containing unsaturated oils and fats, which are particularly prone to oxidation. Synthetic antioxidants are regularly employed to inhibit product oxidation, namely butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or propyl gallate (PG), or to chelate metals and reduce their participation in reactions (e.g.: ethylenediaminetetraacetic acid, EDTA). But despite having a similar biological effect on the molecular, cellular and organ level as natural antioxidants, synthetic antioxidants are also associated with some degree of toxicity and carcinogenicity (Sen and Chakraborty 2011; Sindhi et al. 2013). These safety issues, together with consumers' preferences, has shifted attention to natural antioxidants. It is accepted that plant-based antioxidants, being part of the physiological functions of living organisms, have higher compatibility with the human body (Sen and Chakraborty 2011).

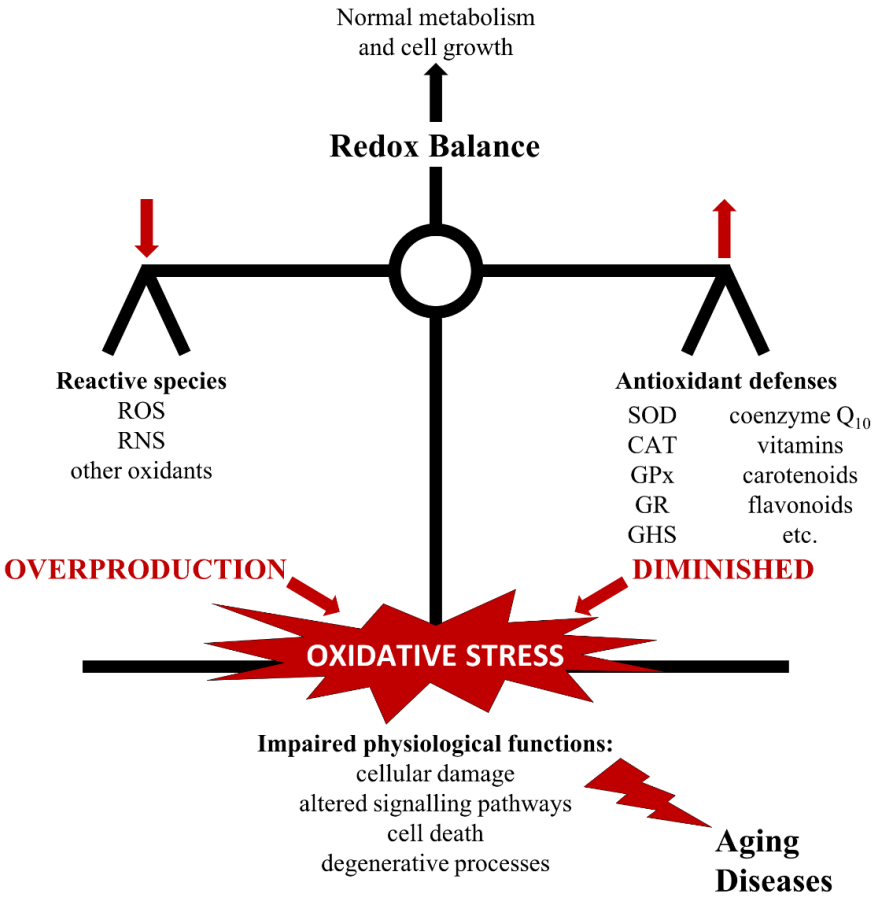

Figure 1.3 Redox balance / imbalance between reactive species and antioxidant defences; oxidative stress and potential deleterious effects.

Extracts and / or compounds from natural sources with strong antioxidant properties are under high demand as alternatives to synthetic antioxidants in the food, pharmaceutical and cosmetic industries and, in this sense, halophytes appear as a very promising and almost untapped source of novel antioxidants. As mentioned before, these plants are exposed to hostile abiotic conditions, very prone to oxidative stress-inducing damage. To survive and flourish in such harsh environments they have developed powerful antioxidant systems that include highly bioactive molecules, many of which are potent antioxidants. In fact, the antioxidant activity is characteristically verified when studying halophytes (Amessis-Ouchemoukh et al. 2014; Jallali et al. 2014; Ksouri et al. 2012; Lopes et al. 2016; Oueslati et al. 2012; Qasim et al. 2016; Petropoulos et al. 2018a,b; Rodrigues et al. 2014, 2015, 2016, 2017a, 2018b; Stanković et al. 2015; Zengin et al. 2018) and, in some species, the antioxidant potential exceeds that of many natural and synthetic antioxidants (e.g.: *T. gallica*, Ksouri et al. 2009; *C. edulis*, Custódio et al. 2012; *S. fruticosa*, *Salvadora persica* L., Qasim et al. 2016).

1.2.2. Anti-diabetic properties

Diabetes mellitus type 2 (T2DM), or non-insulin dependent diabetes, is a common chronic metabolic disorder characterized by high plasma glucose levels (hyperglycaemia) due to improper insulin secretion and / or insulin resistance in peripheral tissues. It is the most common form of diabetes and affects millions of people in both developed and developing countries, being estimated that it will affect roughly 450 million people by 2030; hence, it is considered an emerging health challenge. If left untreated, it can lead to major metabolic complications like retinopathy, neuropathy, accelerated atherosclerosis, and / or renal dysfunction. Currently, T2DM is treated through diet, low in carbohydrates, and oral hypoglycaemic drugs. Different classes of anti-diabetic drugs are available, each with specific action, but one effective therapeutic strategy includes inhibiting carbohydrate-hydrolysing enzymes like α-glucosidase and α-amylase, delaying carbohydrate absorption in the intestinal tract (Hung et al. 2012; Kumar et al. 2011; Panigrahy et al. 2017; Patel et al. 2012). These enzymes catalyse the hydrolytic cleavage of oligosaccharides into absorbable monosaccharides in the small intestine, facilitating glucose absorption. Inhibition of those enzymes decreases the rate of hydrolytic cleavage, which delays the overall glucose absorption into the blood. Therefore, inhibiting carbohydrate-hydrolysing enzymes delays carbohydrate digestion and glucose absorption, resulting in lowered postprandial blood glucose levels and overall reducing hyperglycaemia linked to T2DM (Kumar et al. 2011). Clinically used inhibitors of

carbohydrate-hydrolysing enzymes, such as acarbose, may induce side-effects like abdominal distension and flatulence, reinforcing the need for new anti-diabetic compounds with reduced side-effects (Hung et al. 2012). The World Health Organization (WHO) recommended assessing medicinal plants used to treat diabetes as they can be excellent candidates for effective therapy compounds with less or no side effects. Metformin, for example, is a potent glucose-lowering agent developed from *Galega officinalis* L. and the only medication with approved use in children (Patel et al. 2012). Natural products have great structural diversity and can therefore be a good source for such therapeutic enzyme inhibitors (Kumar et al. 2011). In this sense, highly diverse plants like halophytes can be a great reservoir to search for that type of biologically active compounds.

In T2DM, oxidative stress has been found to mediate the disease's effects. The condition is associated with increased formation of free radicals and decreased antioxidant potential, ultimately resulting in oxidative damage. Oxidative stress has been implicated as a causative factor in the induction and progression of the disease, contributing to the development of diabetes-associated complications (e.g.: cardiovascular, neurological, retinal or urological disorders) (Panigrahy et al. 2017; Patel et al. 2012; Saeidnia and Abdollahi 2013; Sindhi et al. 2013). In this context, antioxidant phytochemicals, well recognized for effectively fighting and preventing oxidative damage, can be used to prevent or manage oxidative-stress related disorders, being positively associated in T2DM management (Saeidnia and Abdollahi 2013; Sindhi et al. 2013). New compounds / products that can include both inhibiting carbohydrate-hydrolysing enzymes and oxidative stress prevention could be a future approach for patients better manage T2DM and related effects. In this sense, medicinal plants with anti-diabetic applications (e.g.: halophytes *A. maritima*, Sheikh et al. 2015; *P. lentiscus*, Bozorgi et al. 2013) and proved hypoglycaemic and antioxidant effects (e.g.: *P. maritimum*, Rodrigues et al. 2017b; *A. macrostachyum*, *H. portulacoides*, *S. europaea*, Zengin et al. 2018) can be vital in the search for better treatment of diabetes and associated complications (Patel et al. 2012).

1.2.3. Anti-hyperpigmentation properties

Nowadays, natural products are in high demand especially for substances with anti-ageing or beauty-enhancement properties. One particular concern in human wellbeing and cosmetics is related to skin hyperpigmentation and agents that promote skin-whitening are increasingly sought after. Skin hyperpigmentation or melanogenic disorders, such as melasma, freckles, solar lentigos, or post-inflammatory hyperpigmentation, are a result of abnormal accumulation

and biosynthesis of melanin (Hakozaki et al. 2015; Khan 2007; Ribeiro et al. 2015). Melanin is produced and distributed in the skin and hair follicles, playing a protective role against UV light-damage. Over-production of melanin can be derived from the over-activity of tyrosinase, a multifunctional copper-containing enzyme that is essential in melanin biosynthesis as it is the first enzyme in the conversion of tyrosine to melanin (Hakozaki et al. 2015; Khan 2007; Ribeiro et al. 2015). Tyrosinase is also responsible for the undesirable browning of fruits and vegetables in post-harvest processing, which decreases their market value (Khan 2007; Chang 2009). In this context, chemicals with the ability to inhibit tyrosinase activity can be used in both the cosmetic and food industries as preventers of hyperpigmentation and enzymatic browning, respectively. Kojic acid, arbutin and hydroquinones are examples of synthetic tyrosinase inhibitors used as anti-hyperpigmenting agents (Chang 2009; Hakozaki et al. 2015; Khan 2007; Ribeiro et al. 2015). But herbal products, in the form of isolated molecules or extracts, are gaining increasing importance as active ingredients particularly in cosmetic formulations due to their recognized protective and skin-treatment features, coupled with their mild and biodegradable characteristics (Chanchal and Swarnlata 2008). Hence, novel anti-hyperpigmentation phytochemicals from natural sources are required not only for cosmetic and pharmaceutical purposes but also for their potential in improving food quality. Plant polyphenols, flavonoids in particular, are reported effective natural tyrosinase inhibitors (Chang 2009; Khan 2007; Ribeiro et al. 2015). Additionally, halophyte plants have been reported to have anti-tyrosinase activities, namely *P. lentiscus* (Lopes et al. 2016) and *A. pungens* (Rodrigues et al. 2018b), proving their potential value in preventing or managing undesired skin and food darkening.

1.2.4. Anti-protozoan properties

Natural products have afforded important antiparasitic compounds and one emblematic example that awarded its discoverer, Youyou Tu, the joint 2015 Nobel Prize of Physiology and Medicine, is the anti-malarial compound artemisinin. Isolated from the plant *Artemisia annua* L., artemisinin established novel therapies for malaria, caused by the protozoan *Plasmodium falciparum*. Before artemisinin, the first effective western treatment for malaria was quinine, isolated from the cinchona tree bark (*Cinchona* sp.) (Efferth et al. 2015). While this reinforces the value of natural resources and their unlimited chemical diversity in finding new or alternative drugs, little is known regarding the potential of halophytes against protozoan infections, such as Chagas disease, caused by *T. cruzi*, or leishmaniasis, caused by *Leishmania*

species. Nevertheless, Oliveira et al. (2016, 2018) screened for *in vitro* anti-*T. cruzi* and anti-*L. infantum* properties in some halophytes, finding activity in *J. acutus* against *T. cruzi* and in *I. crithmoides* and *S. rubra* against *L. infantum*. This demonstrates that halophytes are an overlooked prospective source of anti-protozoan molecules.

1.3. Preliminary toxicity screening

The potential toxicity of new compounds or products (herbal extracts, for example) for human use must be ascertained to establish its safe consumption. In this sense, cytotoxicity assays are common *in vitro* methods employed as preliminary toxicological assessment for substances, adopted for safety evaluation in numerous national and international standards. Assessing cytotoxicity of plant extracts and / or substances is usually accomplished through *in vitro* cell culture models using different cancer and non-cancer mammalian cell lines to assess endpoints of cellular viability such as metabolic activity and plasma membrane integrity following exposure to a test extract / compound. These assays are useful not only to determine potential and selective anti-tumoural activities, but also to predict potential toxicity of those extracts / compounds (Liu et al. 2018; Nogueira et al. 2011). Current approaches to measure the degree of potential toxicity include assays that evaluate various aspects of cell viability, and one of the most commonly used techniques is the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a colorimetric method to determine cell metabolic state. Preliminary toxicity screenings provide fast, standardized, sensitive, economical and reliable results to determine whether a substance contains potentially toxic activity (Liu et al. 1997; Nogueira et al. 2011; Saad et al. 2006). They also reduce *in vivo* testing, since a positive correlation has been established between *in vitro* toxicity towards mammalian cell lines and *in vivo* toxicity determined in animal models (Carballo et al. 2002; Parra et al. 2001). Overall, a decreased or absent cytotoxic effect when assessing herbal extracts / products suggests that they can be considered non-toxic and safe for consumption. Moreover, the fact that a species is widely used in traditional medicine and / or for nutritional purposes is by itself an indication of its low toxicity.

1.4. Plant Metabolites

Plants have a high biosynthetic capacity, being able to generate a broad spectrum of metabolites generally classified as primary or secondary, depending on their role in plant

metabolism. Primary metabolites are produced within primary biosynthetic and metabolic routes aimed at plant growth and development and are vital for the plant's functioning; they comprise carbohydrates, lipids, amino acids, proteins, and nucleic acids, and are found across all species within broad phylogenetic groups. Secondary metabolites are not directly needed for growth and development although holding important functions; they include alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived from carbohydrates) and are often differentially distributed among taxonomic groups. They are important in mediating a vast array of interactions between plants and their environment, determining colour, acting as defensive agents against pathogens, insects and herbivores, providing reproductive advantages to attract pollinators and seed dispersers, inhibiting the growth of surrounding competitor plants, acting as cellular signalling molecules, or protecting against oxidative damage. These plant constituents are in fact the major source of antioxidants as the majority of these phytochemicals are redox active molecules. The biosynthesis and accumulation of this wide array of compounds provides unique chemical structures with unusual biological activities. While some primary metabolites can also show relevant biological properties (e.g.: carbasugars, a class of carbohydrates, exhibit various bioactivities including antimicrobial and glycosidase inhibition; the most well-known carbasugar is the anti-influenza oseltamivir phosphate [Tamiflu®]; Usami 2014), secondary metabolites are usually the bioactive compounds, some of them responsible for the health benefits attributed to botanical extracts and displaying interesting pharmacological activities (Bernhoft 2010; Hounsome et al. 2008; Saeidnia and Abdollahi 2013; Sen and Chakraborty 2011).

1.4.1. Bioactive secondary compounds

More than 200 000 secondary metabolites are known, with many more yet to be discovered, providing mankind with a natural reservoir of potential pharmaceuticals. Typically, bioactive compounds in plants are produced as secondary metabolites. They can be defined as substances that elicit an effect (pharmacological or toxicological) on biological systems. There is no commonly agreed system of classification of active constituents, but they can be divided, for example, according to biosynthetic pathways. Based on their biosynthetic origins, the main chemical groups of bioactive compounds can be classified as terpenes and terpenoids, produced through the mevalonic acid pathway and non-mevalonate (MEP) pathway, alkaloids, produced through the shikimic acid pathway and the tricarboxylic acid cycle, and phenolic compounds,

synthesized through the shikimic and malonic acids pathways (Figure 1.4) (Azmir et al. 2013; Bernhoft 2010; Doughari 2012; Hounsome et al. 2008; Ncube and Staden 2015).

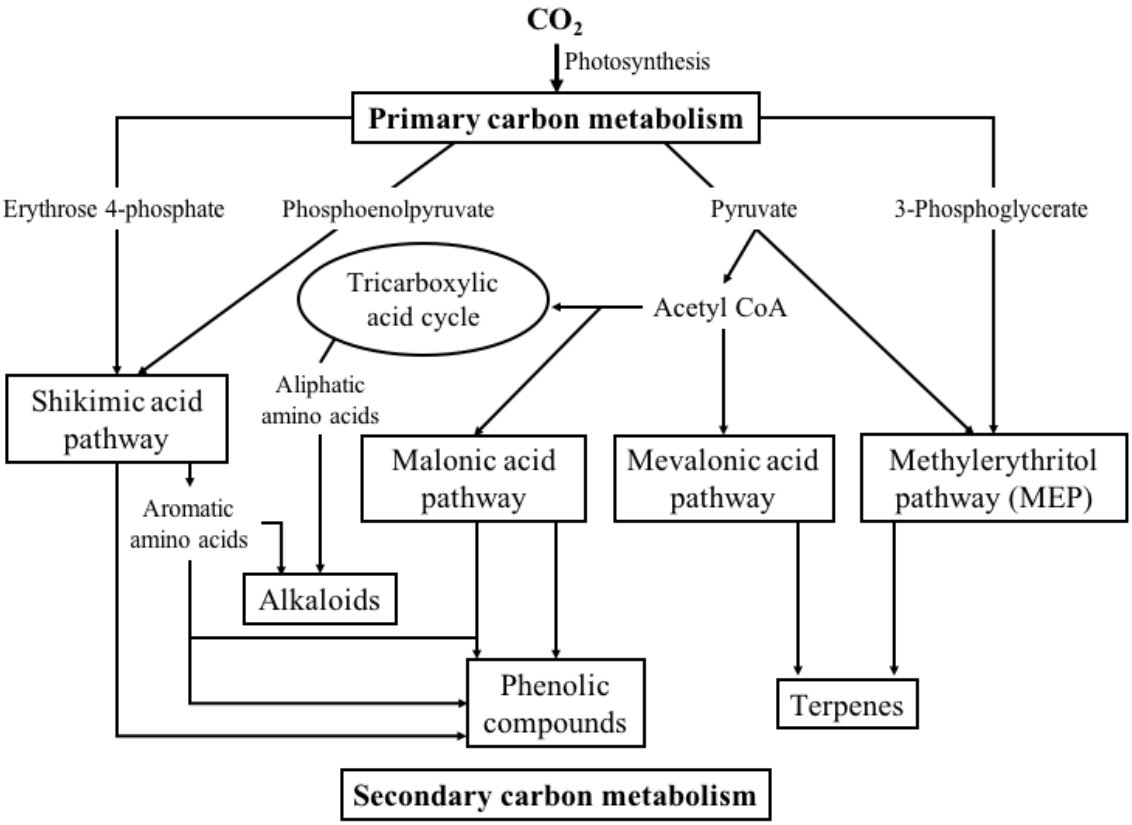

Figure 1.4 A simplified general overview of the biosynthetic pathways involved in the biosynthesis of the three major groups of plant bioactive compounds (adapted from Azmir et al. 2013).

1.4.1.1. Terpenes and terpenoids

Terpenes and terpenoids are among the most structurally diverse groups of secondary metabolites, derived by repetitive fusion of branched 5-carbon isoprene units. They are synthesised from two precursors, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), through two independent pathways, the mevalonate pathway that provides the precursors for sesquiterpenes and sterols, and the non-mevalonate pathway (MEP) in plastids that furnishes monoterpenes, diterpenes, and carotenoids. Their chemical diversity is probably a reflection of their numerous biochemical functions, such as electron carriers (ubiquinone, plastoquinone), components of membranes (sterols), photosynthetic pigments (carotenoids), hormones (gibberelins, abscisic acid), plant defence, pollinators' attractants, etc. Examples of commonly known monoterpenes are camphor and menthol; diterpenes are usually considered resins; triterpenes include steroids and sterols; sesquiterpenes, like monoterpenes,

are major components of many essential oils. Major dietary terpenoids include carotenoids, tocopherols and tocotrienols, quinones, and sterols. Additionally, they also show biological activities such as antimicrobial, insecticidal, anti-inflammatory, sedative, or cytotoxic. Pharmaceuticals derived from terpenes include artemisinin, an anti-malarial sesquiterpenoid isolated from *A. annua*, taxol, a high-value diterpenoid-derived anti-cancer drug from the bark of *T. brevifolia*, or azadirachtin A, an insect antifeedant terpenoid isolated from *Azadirachta indica* A.Juss. (Doughari 2012; Hounsome et al. 2008; Ksouri et al. 2012; Ncube and Staden 2015).

1.4.1.2. Alkaloids

Alkaloids are one of the largest groups of secondary metabolites comprised by nitrogen-containing molecules synthesized mainly from amino acids, although some purine-derived alkaloids are also known, with various radicals replacing one or more of the hydrogen atoms in the peptide ring. They are produced through the shikimic acid pathway with aromatic amino acids as starting precursor molecules, and through tricarboxylic acid cycle from aliphatic amino acids. These compounds present many structures with notable complexity and, although their exact roles are not well understood, they are believed to play an important ecological role enabling plants to interact defensively with the environment. Additionally, they often display important pharmacological properties. In fact, many of them are among the most worldwide used drugs: the analgesics morphine and codeine, the muscle relaxant tubocurarine, the vasodilating agents vincamine and ajmalicine, the anticancer agent vinblastine, or galanthamine for Alzheimer's disease, to name a few. Other well-known alkaloids of plant origin include the stimulants caffeine, nicotine, atropine, and cocaine (Azmir et al. 2013; Doughari 2012; Hounsome et al. 2008; Ksouri et al. 2012; Ncube and Staden 2015).

1.4.1.3. Phenolic compounds

Phenolic compounds, or polyphenols, are perhaps the most widely occurring group of secondary metabolites. They are comprised by at least one aromatic ring bearing one or more hydroxyl substituents and other functional derivatives (e.g. esters, methyl esters, glycosides), which confers structural diversity to the group. The wide range of resulting compounds can be categorised into subgroups (Table 1.2) according to its structural components; from these subgroups, phenolic acids, flavonoids and tannins are considered the main dietary polyphenols (Balasundram et al. 2006; Ncube and Staden 2015). Although ubiquitous in the plant kingdom, the type of polyphenol produced varies greatly between genera and species (Ncube and Staden

2015). In fact, the ability to synthesize phenolic compounds has been naturally selected through evolution in different plant lineages according to specific needs, therefore allowing plants to adapt to different habitats and ever-changing environmental characteristics (Lattanzio 2013).

Table 1.2 Classes of phenolic compounds in plants (Soto et al. 2015).

Classes of phenolic compounds	Examples of compounds in the respective class
Simple phenols	
Phenolic acids	
Hydroxybenzoic acids	Gallic, gentisic, protocatechuic, syringic acids
Hydroxycinnamic acids	Caffeic, *p*-coumaric, ferulic, sinapic acids
Coumarins	Esculetin, scopoletin, umbelliferone
Polyphenols	
Flavonoids	
Flavonols	Kaempferol, myricetin, quercetin
Flavones	Apigenin, luteolin, tangeretin
Isoflavones	Daidzein, genistein
Flavanones	Eriodictyol, hesperetin, naringenin, neohesperidin
Flavanols (flavan-3-ols)	Catechin, epicatechin, epigallocatechin, gallocatechin
Anthocyanins	Delphinidin, cyanidin, malvidin, pelargonidin, petudinin
Chalcones	Arbutin, phloretin, phloridzin
Lignans	Matairesinol, pinoresinol, secoisolariciresinol
Stilbenes	Piceid, pinosylvin, resveratrol
Tannins	Ellagitannins, gallotannins, proanthocyanidins

Plant phenolics derive either from the shikimate / phenylpropanoid pathway, providing phenylpropanoids, or the "polyketide" acetate / malonate pathway, producing simple phenols, or both. These monomeric and polymeric phenols and polyphenols produced fulfil a very broad range of roles in plants (Lattanzio 2013). They are physiologically and morphologically important, playing significant roles in many functions: they provide structure in cell walls, protect from ultraviolet radiation, act as feeding deterrents for many herbivores, defend against pathogens and parasites, while also contributing to colour and sensory properties that attract pollinators, promoting the plant's reproduction (Balasundram et al. 2006; Dai and Mumper 2010; Hounsome et al. 2008; Soto et al. 2015). Phenolic compounds also display free radical-scavenging activity, determined largely by their reactivity as hydrogen- or electron- donating agents, and the stability of the resulting antioxidant-derived radical prevents oxidation (Ncube and Staden 2015). This antioxidant activity depends on the compounds' structure, in particular

on the number and positions of the hydroxyl groups in relation to the carboxyl functional group, and the nature of substitutions on the aromatic rings (Balasundram et al. 2006; Ksouri et al. 2012). Besides their powerful antioxidant properties, which by itself attracts considerable interest, these compounds also exhibit many other biological effects of great importance to human health (Dai and Mumper 2010; Ksouri et al. 2012). They were found to modulate the activity of a wide range of enzymes, cell receptors, and cell signalling pathways, which credits them not only with the prevention of oxidative-stress related conditions (like aging and cardiovascular and neurodegenerative disorders, for example), but also with anti-tumoural, anti-inflammatory, anti-diabetic, antimicrobial, anti-histaminic, or even immuno-modulatory, vasodilator and anti-obesity activities (Balasundram et al. 2006; Dai and Mumper 2010; Hounsome et al. 2008; Ksouri et al. 2012; Ncube and Staden 2015). For example, salicylic acid has analgesic, antipyretic, anti-inflammatory, keratolytic, and other properties associated to it (Hounsome et al. 2008; Khadem and Marles, 2010). This hydroxybenzoic acid was first isolated from the willow bark (*Salix* spp.) in pursuit of the active ingredient responsible for the tree's healing properties. Soon after, acetylsalicylic acid (aspirin) was synthesized with the same efficacy but less irritation to the gastric mucosa, rapidly becoming one of the world's best-selling drugs (Raskin 1992). Catechins (catechin, epicatechin and gallates of epicatechin), the main polyphenols present in tea (*Camellia sinensis* L. (Kuntze)), have been implicated in many of its health-promoting benefits (cancer prevention, cardiovascular risk reduction, anti-diabetic and anti-obesity properties, etc.), particularly epigallocatechin-3-gallate that is the major tea catechin (Khan and Mukhtar 2007). Catechins are examples of flavonoids used in the food industry as natural antioxidants against lipid oxidation of oils and fats and as antimicrobial agents. Additionally, they are also used as health functional ingredient in foods, dietary supplements, and animal feeds (El Gharras 2009). Ferulic acid is also used in the food industry as food additive / preservative, nutraceutical, and functional ingredient, while also pharmacologically used as a photoprotective constituent in sunscreens and skin lotions and as a cross-linking agent to increase viscosity and form gels from some polysaccharides. This almost ubiquitous phytochemical is present in numerous foods (from rice, wheat, oats, grains, beans, fruits and vegetables to seeds, nuts, herbs and spices) and exhibits a wide variety of biological activities (antioxidant, anti-diabetic, anticarcinogenic, cardio- and neuroprotective, anti-inflammatory, antimicrobial, etc.) (Kumar and Pruthi 2014; Ou and Kwok 2004). Another phenolic compound associated to the health benefits of a food product is chlorogenic acid. This major component of coffee is considered a promising nutraceutical and food additive attending to its multifunctional properties, namely antioxidant, antimicrobial, anti-diabetic, anti-obesity,

anti-inflammatory, and anti-hypertensive activities (Meng et al. 2013; Santana-Gálvez et al. 2017). Many other phytochemicals could be added as examples of bioactive compounds essential to the pharmaceutical, cosmetic and / or food industries and as dietary constituents with health-promoting benefits for both human and animal welfare. Most importantly, there are still many more compounds / activities / applications to be uncovered.

1.4.1.3.1. Phenolic compounds in halophytes

Phenolic compounds in plants have a key role in dealing with environmental stresses such as intense light, extreme temperatures, water scarcity, and / or salinity. These challenging conditions lead to increased production of free radicals and other oxidative species, to which plants respond by increasing their capacity to scavenge them, namely synthesizing antioxidant phenolic compounds. In fact, a positive correlation between abiotic stress and increasing polyphenolic contents has been demonstrated in many halophytes (Buhmann and Papenbrock 2013; Hounsome et al. 2008; Lattanzio 2013; Soto et al. 2015). In this sense, due to the harsh conditions in which they survive and thrive, halophytes are a great source of phenolics.

Numerous phenolic compounds have been described in halophyte species, many of them conferring the plants with medicinal properties due to their biological activities (Ksouri et al. 2012). One of the most relevant biological properties of phenolics is antioxidant activity; a myriad of studies have reported them as radical scavengers, hydrogen and electron donors, singlet oxygen quenchers, and / or metal chelators (Balasundram et al. 2006; Ksouri et al. 2012). For example, the flavonol glycoside isorhamnetin 3-*O*-β-$_{D}$-glucopyranoside, isolated from *Salicornia herbacea* (syn. *S. europaea*), successfully scavenged radicals in both cellular-free and cellular-based systems, and significantly elevated cellular glutathione levels and expression levels of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and heme oxygenase-1 (Kong et al. 2009). In *Tamarix boveana* Bunge, the flavanone naringenin was isolated from the aerial-parts' methanolic extract (ethyl acetate fraction) and identified as the molecule responsible for the detected antioxidant properties (Naija et al. 2014). Chlorogenic acid was another phenolic compound established as an effective antioxidant compound in *C. maritimum* (Mekinić et al. 2016), esculetin and luteolin 7-*O*-rutinoside were the main antioxidant components in *Artemisia montana* (Nakai) Pamp. (Buhmann and Papenbrock 2013), and rutin and apigenin were the most active radical scavengers in *Teucrium polium* L. (Sharififar et al. 2009).

Phenolic compounds also display other important biological properties, apart from antioxidant. For example, Gálvez et al. (2003) identified the flavone luteolin-7-*O*-β-glucoside,

the major flavonoid in *Plantago* species, as the molecule responsible for the cytotoxic capacity of leaves' methanolic extracts on breast and renal adenocarcinomas and melanoma cell lines. Caffeic and chlorogenic acids were identified among 11 pure compounds previously reported in *P. major* as the molecules with strongest antiviral activity against some herpes simplex and adenovirus types (Ksouri et al. 2012). Two flavonol glycosides isolated in *S. herbacea*, isorhamnetin 3-O-β-$_D$-glucopyranoside and quercetin 3-O-β-$_D$-glucopyranoside, effectively suppressed adipogenic differentiation in mouse 3T3-L1 preadipocyte cells, proving to be potent anti-obesity agents via alleviation of lipid accumulation (Kong et al. 2012). Additionally, several studies assessed the *in vitro* bioactivities of extracts from different halophyte species, along with its major constituents, associating the displayed activities to already known properties of the main compounds. *Atriplex halimus*, for instance, has its potent antioxidant activity attributed to the presence of flavonols common in *Atriplex* species such as kaempferol, quercetin, or isorhamnetin (Ksouri et al. 2012). *Cynara cardunculus* leaves' methanolic extracts exhibited activity against human pathogenic bacteria (e.g. *Staphylococcus aureus*, *Escherichia coli*), possibly due to the predominant phenolics identified in the extracts, namely syringic and transcinnamic acids, epicatechin and quercitrin (Falleh et al. 2008). The major constituents of *Artemisia scopariae* Waldst. et Kit. are *p*-hydroxyacetophenone, chlorogenic and caffeic acids, which are probably responsible for the antioxidant, anti-inflammatory, antitumor and antibacterial properties of the plant (Ksouri et al. 2012). However, one must keep in mind that while the properties of individual compounds can be specifically determined, the bioactivities displayed by extracts made from halophyte species (and other organisms) can also be the result of synergistic and / or additive effects of the phytoconstituents' mixture in those extracts. On the other hand, one single compound can be responsible for multiple bioactivities (Buhmann and Papenbrock 2013).

1.5. Halophytes from the Algarve Coast, Southern Portugal

Considerable biodiversity in terms of halophyte species occurs in southern Portugal, some with known therapeutic applications (e.g.: *A. donax*, *C. coronopifolia*, *P. lentiscus*, *R. raphanistrum*; *S. fruticosa*, *S. ramosissima*, see Table 1.1; Carapeto 2006), but reports of their bioactivities are particularly scarce. In fact, few species have been investigated despite their high potential as source of bioactive molecules. Some halophytes from the Algarve coast have recently been studied showing promising results in terms of antioxidant, neuroprotective, anti-inflammatory, anti-diabetic, anti-melanogenic and anti-tumoural activities (see section 1.2

Biological Activities of Halophytes). Still, significant part of the species in this area, many with medicinal uses, has never been tested for biological activities or evaluated for phytochemical composition. In fact, medicinal halophytes remain largely unexplored and underutilized despite their outstanding potential as a reservoir of bioactive compounds, drugs, novel food-products and innovative health-promoting products, or as raw material for the pharmaceutical, cosmetic and / or food industries. Indeed, their ethnopharmacological uses suggest a high ethnic and traditional value still untapped.

This work focuses on five halophyte plants common in the Algarve, chosen for their ethnopharmacological value in the Mediterranean region and potential bioactivities: *Artemisia campestris* subsp. *maritima*, *Crithmum maritimum*, *Eryngium maritimum*, *Helichrysum italicum* subsp. *picardii*, and *Plantago coronopus*, whose brief description follows next.

1.5.1. Halophytes used in this study

1.5.1.1. *Artemisia campestris* L. subsp. *maritima* Arcangeli

Figure 1.5 Specimens of *A. campestris* subsp. *maritima* in the field with detail of the leaves (photos from http://flora-on.pt).

Taxonomic classification*	Kingdom: Plantae Division: Spermatophyta Subdivision: Magnoliophytina Class: Magnoliopsida Sub-classe: Asteridae Order: Asterales Family: Asteraceae Genus: *Artemisia* Species: *Artemisia campestris* L. Sub-species: *Artemisia campestris* L. subsp. *maritima* Arcangeli
Synonyms	Artemisia *crithmifolia* L.
Common name	Dune wormwood, "madorneira" or "erva-lombrigueira" in Portugal
Habitat	Coastal sand dunes; perennial
Distribution	Temperate European Atlantic coast (Almargem 2018; Dib et al. 2016)
Medicinal uses	To treat gastric disorders, hypertension and rheumatics; anthelmintic and abortifacient properties (Almargem 2018). The species, *A. campestris*, is also described as having anti-diabetic, anti-inflammatory and antipyretic uses (Dib et al. 2016).
Used formulations	Herbal teas made from stems and leaves (Almargem 2018).
Biological activities	Antioxidant and anti-microbial activities (Megdiche-Ksouri et al. 2015).
Bioactive compounds	Phenolic acids, flavonoids, coumarins, sesquiterpenes and acetophenone derivatives (Rauter et al. 1989; Sanz et al. 1991; Vasconcelos et al. 1998; Megdiche-Ksouri et al. 2015).

*according to Checklist da Flora de Portugal (2010)

1.5.1.2. *Crithmum maritimum* L.

Figure 1.6 Specimens of *C. maritimum* in the field (Martinhal beach, Sagres, south Portugal), with detail of the flowers (photos by the author).

Taxonomic classification*	Kingdom: Plantae Division: Spermatophyta Subdivision: Magnoliophytina Class: Magnoliopsida Sub-classe: Rosidae Order: Umbellales Family: Apiaceae Genus: *Crithmum* Species: *Crithmum maritimum* L.
Synonyms	-
Common name	Sea fennel, rock samphire, "funcho marítimo" in Portugal
Habitat	Rocky shores and coastal cliffs; perennial
Distribution	European and North-African Atlantic, Mediterranean and Black Sea coasts (Atia et al. 2011; Castroviejo et al. 2003)
Medicinal uses	Described as appetizer, tonic, purgative, carminative, anthelmintic, diuretic; used to prevent scurvy and to treat renal and urinary complaints, digestive disorders, colic and inflammation of the urinary tract and prostate (Atia et al. 2011; Cornara et al. 2009; Franke 1982).
Used formulations	Infusions and decoctions of aerial parts (Atia et al. 2011; Cornara et al. 2009; Franke 1982).
Biological activities	Antioxidant and antimicrobial activities (Atia et al. 2011; Buhmann and Papenbrock 2013).
Bioactive compounds	Phenolic acids and flavonoids (Atia et al. 2011; Buhmann and Papenbrock 2013).

*according to Checklist da Flora de Portugal (2010)

1.5.1.3. *Eryngium maritimum* L.

Figure 1.7 Specimens of *E. maritimum* in the field (Amoreira beach, Aljezur, south Portugal) and after collection, with detail of the flowers (photos by the author).

Taxonomic classification*	Kingdom: Plantae Division: Spermatophyta Subdivision: Magnoliophytina Class: Magnoliopsida Sub-classe: Rosidae Order: Umbellales Family: Apiaceae Genus: *Eryngium* Species: *Eryngium maritimum* L.
Synonyms	-
Common name	Sea holly, "cardo marítimo" in Portugal
Habitat	Dunes and sandy beaches; perennial
Distribution	Mediterranean, Black Sea, Atlantic and Baltic coasts; introduced in eastern North America and Australia (Castroviejo et al. 2003; Isermann and Rooney 2014)
Medicinal uses	Used as diuretic, kidney stone inhibitor, aphrodisiac, expectorant, anthelmintic, antitoxin against various infections, for oedema reabsorption and / or pain relief (Erdem et al. 2015; Isermann and Rooney, 2014; Lisciani et al. 1984). *Eryngium* species also have uses like hypoglycaemic, anti-inflammatory, poison antidote, stimulant and antitussive, among others (Erdem et al. 2015; Wang et al. 2012).
Used formulations	Tinctures, infusions and decoctions of whole plant, including roots (Hool 1922).
Biological activities	Antimicrobial, antioxidant and / or anti-inflammatory activities (Amessis-Ouchemoukh et al. 2014; Conea et al. 2016; Darriet et al. 2014; Erdem et al. 2015; Kholkhal et al. 2012; Lisciani et al. 1984; Mejri et al. 2017; Meot-Duros et al. 2008; Rjeibi et al. 2017; Wang et al. 2012; Yurdakok et al. 2014).
Bioactive compounds	Phenolic acids, flavonoids and terpenes (Amessis-Ouchemoukh et al. 2014; Conea et al. 2016; Darriet et al. 2014; Erdem et al. 2015; Kholkhal et al. 2012; Mejri et al. 2017; Rjeibi et al. 2017; Wang et al. 2012).

*according to Checklist da Flora de Portugal (2010)

1.5.1.4. *Helichrysum italicum* (Roth) G. Don subsp. *picardii* (Boiss & Reuter) Franco

Figure 1.8 Specimens of *H. italicum* subsp. *picardii* in the field (Ancão beach, Loulé, south Portugal), with detail of the flowers (photos by the author).

Taxonomic classification*	Kingdom: Plantae Division: Spermatophyta Subdivision: Magnoliophytina Class: Magnoliopsida Sub-classe: Asteridae Order: Asterales Family: Asteraceae Genus: *Helichrysum* Species: *Helichrysum italicum* (Roth) G. Don Sub-species: *Helichrysum italicum* (Roth) G. Don subsp. *picardii* (Boiss & Reuter) Franco
Synonyms	*Helichrysum picardii* Boiss. & Reuter
Common name	Everlasting, "perpétua das areias" or "erva-caril" in Portugal
Habitat	Dunes and sandy beaches; perennial
Distribution	Southern Europe (Bingre et al. 2007; Viegas et al. 2014)
Medicinal uses	Associated to analgesic properties and used in dermatologic, respiratory and digestive disorders with inflammatory, allergic or infectious components (Facino et al. 1990; Viegas et al. 2014). *Helichrysum* plants are also used as diuretic and to treat urinary disorders, burns, venomous bites and hernias (Viegas et al. 2014).
Used formulations	Infusions and decoctions of whole plant (Facino et al. 1990; Bingre et al. 2007; Viegas et al. 2014).
Biological activities	Antimicrobial and anti-inflammatory properties (Facino et al. 1990; Viegas et al. 2014).
Bioactive compounds	Different classes of bioactive molecules, the most common being phenolic compounds and terpenes (Facino et al. 1990; Maksimovic et al. 2017; Viegas et al. 2014).

*according to Checklist da Flora de Portugal (2010)

1.5.1.5. *Plantago coronopus* L.

Figure 1.9 Specimens of *P. coronopus* in the field (Ludo area in Ria Formosa lagoon, Olhão, south Portugal), with detail of the flowers (photos by the author).

Taxonomic classification*	Kingdom: Plantae Division: Spermatophyta Subdivision: Magnoliophytina Class: Magnoliopsida Sub-classe: Asteridae Order: Plantaginales Family: Plantaginaceae Genus: *Plantago* Species: *Plantago coronopus* L.
Synonyms	-
Common name	Buckshorn plantain, "diabelha" in Portugal
Habitat	Saline or disturbed areas, mainly in coastal habitats; annual or biennial
Distribution	Mediterranean and Atlantic-European areas, North-west Africa and West Asia; introduced in North America, Australia and New Zealand (Almargem 2018; Castroviejo et al. 2009).
Medicinal uses	Described uses as analgesic, anti-inflammatory, antipyretic, anticancer, emollient and to treat the respiratory system (Ksouri et al. 2012; Redzic 2006). *Plantago* species are also used for their antimicrobial, astringent, expectorant and diuretic properties (Gonçalves and Romano 2016; Ksouri et al. 2012).
Used formulations	Infusions, decoctions and tinctures of roots, leaves and / or flowers (Aflosul 2006; Carvalho 2006; Redzic 2006; Neves et al. 2009)
Biological activities	Cytotoxic, anti-inflammatory and antioxidant properties (Gálvez et al. 2003, 2005; Rodrigues et al. 2014).
Bioactive compounds	Phenolic compounds and glycosides (Gálvez et al. 2003, 2005; Grubešić et al., 2013; Janković et al. 2012).

*according to Checklist da Flora de Portugal (2010)

1.6. Scope and Main Objectives of this Study

The main scope of this study was to expand our limited knowledge on the potential therapeutic and health-promoting properties of medicinal halophytes.

Although halophyte chemistry implies them as sources of both novel and known compounds, they are still largely unexplored, and reports of their bioactivities are scarce, namely with halophytes from southern Portugal (Algarve). Medicinal halophytes in particular, considering their ethnopharmacological use, can be a reservoir of new compounds and applications. Hence, this study intended to explore the potential of medicinal halophytes from the Algarve coast as sources of bioactive compounds with therapeutic application, validating their uses in folk medicine and searching for new bioactivities and thereby answering the following biological questions:

- Can the traditional use of halophytes in folk medicine be supported by scientific evidence?
- Do these halophytes and / or their extracts have potential application as therapeutic, health-promoting or food-additive commodities?
- Can these plants be good candidates for identification and commercialization of bioactive compounds and / or nutraceuticals from natural sources?

The author believes that the biological and chemical characterization of these halophytes is bound to produce an array of extracts and / or compounds with potential application as health promoting commodities.

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CHAPTER 2

PROFILING OF ANTIOXIDANT POTENTIAL AND PHYTOCONSTITUENTS OF *PLANTAGO CORONOPUS*

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Profiling of antioxidant potential and phytoconstituents of *Plantago coronopus*

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Abstract

The halophyte species *Plantago coronopus* has several described ethnomedicinal uses, but few reported biological activities. This work carried out for the first time a comparative analysis of *P. coronopus* organs in terms of phenolic composition and antioxidant activity of organic and water extracts from roots, leaves and flowers. The leaves contents in selected nutrients, namely amino acids and minerals, are also described. Roots (ethyl acetate and methanol extracts) had the highest radical scavenging activity (RSA) towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals, while leaves (hexane extract) had higher RSA on nitric oxide radical and iron chelating ability. High performance liquid chromatography (HPLC) analysis identified eighteen phenolics from which salicylic acid and epicatechin are here firstly described in *Plantago* species. Leaves had mineral levels similar to those of most vegetables, proving to be a good source for elements like calcium, sodium, iron and magnesium, and also for several of the essential amino acids justifying it use as food. Our results, especially those regarding the phenolics composition, can explain the main traditional uses given to this plantain and, altogether, emphasize the potential of *P. coronopus* as a source of bioactive molecules particularly useful for the prevention of oxidative stress-related diseases.

Keywords: Plantago coronopus, halophytes, phytoconstituents, phenolics, antioxidant activity.

Avaliação do potencial antioxidante e perfil fitoquímico do *Plantago coronopus*

Resumo

A espécie halófita *Plantago coronopus* tem vários usos etnomedicinais já descritos, mas em relação à bioatividade a informação é escassa. Este trabalho efetuou, pela primeira vez, uma análise comparativa dos órgãos de *P. coronopus* em termos de compostos fenólicos e atividade antioxidante de extratos orgânicos e aquosos provenientes das raízes, folhas e flores da planta, bem como o conteúdo de determinados nutrientes, aminoácidos e minerais, nas folhas da planta. As raízes (extratos de acetato de etila e metanol) apresentaram a maior atividade de captação para os radicais 1,1-difenil-2-picril hidrazil (DPPH) e 2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfónico) (ABTS), enquanto as folhas (extrato de hexano) mostraram maior atividade captadora para o radical óxido nítrico bem como maior capacidade quelante do ferro. A análise por cromatografia liquida de alta eficiência (CLAE) identificou dezoito compostos fenólicos e, destes, o ácido salicílico e a epicatequina são aqui descritos pela primeira vez em espécies de *Plantago*. As folhas desta planta halófita mostraram ainda conter minerais em níveis semelhantes aos da maioria dos vegetais, provando ser uma boa fonte de elementos como o cálcio, sódio, ferro e magnésio, bem como de vários dos aminoácidos essenciais o que justifica seu uso na alimentação. Os resultados, particularmente aqueles relacionados à composição fenólica, podem justificar os principais usos medicinais atribuídos a esta espécie e, na sua totalidade, demonstram o potencial de *P. coronopus* como fonte de moléculas bioativas particularmente úteis na prevenção de doenças relacionadas com estresse oxidativo.

Palavras-chave: Plantago coronopus, halófitas, fitoconstituintes, fenólicos, atividade antioxidante.

1. Introduction

Plantago coronopus, commonly known as buckshorn plantain ("diabelha" in Portugal), is a medicinal halophyte belonging to the genus *Plantago* L., the largest of the Plantaginaceae family (Castroviejo et al., 2009; Ksouri et al., 2012). This salt-tolerant worldwide-distributed herbaceous plant, usually annual or biennial, grows on saline or disturbed areas, mainly in coastal habitats where it is exposed to fluctuating environmental conditions (Castroviejo et al., 2009). *P. coronopus* is edible and cooked as a vegetable in Balkan traditional cuisine, its leaves are salad ingredients in France and Italy, and it is a potentially important cash crop for animal feeding (Gálvez et al., 2005; Redzic, 2006).

In folk medicine *Plantago* species are widely used, for example, for their anticancer, antimicrobial, anti-viral, anti-inflammatory, analgesic, astringent, expectorant and diuretic properties (Gonçalves and Romano, 2016; Ksouri et al., 2012). *P. coronopus* in particular has several described ethnomedicinal uses in several countries, such as analgesic, anti-inflammatory, antipyretic, anticancer, emollient and to treat the respiratory system (Ksouri et al., 2012; Redzic, 2006). Several *Plantago* species display an array of bioactivities such as antioxidant, cytotoxic, anti-inflammatory and antiviral that can account for their medicinal uses (Beara et al., 2009, 2012a, b; Gálvez et al., 2003, 2005; Gonçalves and Romano, 2016; Ksouri et al., 2012). In fact, this genus contains a high amount of primary and secondary metabolites like phenolic acids, flavonoids, coumarins, lignans, glycosides, triterpenes and polysaccharides that can be responsible for those bioactivities (Beara et al., 2009, 2012a, b; Gonçalves and Romano, 2016; Jurišić Grubešić et al., 2013; Janković et al., 2012; Ksouri et al., 2012). Moreover, it is known that other *Plantago* species, namely *P. major*, *P. lanceolata* and *P. media* are valuable sources of amino acids and minerals, which may contribute for their use as an ingredient in human nutrition (Guil-Guerrero, 2001; Amaglo et al., 2010).

Reports on biological activities of *P. coronopus* are scarce and include cytotoxic, anti-inflammatory and anti-radical properties mainly of leaf extracts (Gálvez et al., 2003, 2005; Rodrigues et al., 2014). Some phenolic compounds and glycosides are described in leaves or aerial parts of this species (Gálvez et al., 2003, 2005; Jurišić Grubešić et al., 2013; Janković et al., 2012). Also, comparative studies of the biological activities of different anatomical organs of this species were not found. Therefore, considering the high potential of *Plantago* species as a source of biologically active compounds and the lack of information regarding *P. coronopus*, this work aimed to evaluate the antioxidant potential and phenolic composition of organic and water extracts from roots, leaves and flowers of *P. coronopus*, along with the amino acid and mineral content of leaves. This report could provide useful knowledge for the development of high added value health promoting commodities from *P. coronopus*.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of analytical grade. Reagents 1,1-diphenyl-2picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), ethylenediamine tetraacetic acid (EDTA), pyrocatechol violet, sodium nitrite, aluminium chloride, butylated hydroxytoluene (BHT), and all commercial standards were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied phosphoric acid and Folin-Ciocalteau phenol reagent. Additional reagents and solvents were obtained from VWR International (Belgium).

2.2. Plant collection

Whole plants of *Plantago coronopus* were collected in Ludo, Ria Formosa, a coastal lagoon in south Portugal, in June 2013 (37º 01' 14.2" N 7º 53' 05.5"¨W), and its taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). A voucher specimen is kept in the herbarium of the Marbiotech Laboratory (voucher MBH02). Plants were divided in roots, leaves and flowers, oven dried for 3 days at 50 °C, milled and stored at –20 °C until use.

2.3. Extracts preparation

Dried biomass was first mixed with hexane (1:10, w/v), using a disperser (2 min, IKA T10B Ultra-Turrax) for cell disruption, vortexed for 1 min at room temperature (RT) and centrifuged (6000 *g*, 10 min). Extraction was repeated three times, and the supernatants combined and filtered (Whatman no. 4). Pellets were then extracted sequentially with solvents of increasing polarity, namely ethyl acetate, methanol and water. All the extracts were vacuum-dried and re-suspended in the corresponding solvent and/or dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL and stored at –20 °C.

2.4. Phytochemical composition of the extracts

2.4.1. Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content

TPC was determined by the Folin-Ciocalteau assay according to Velioglu et al. (1998), adapted to 96-well plates (described in Rodrigues et al., 2015). Absorbance was measured at 725 nm using gallic acid as standard and results were expressed as milligrams of gallic acid equivalents per grams of biomass dried weight (mg GAE/g dw). TFC was estimated by the aluminium chloride colorimetric method, adapted to 96-well plates (Zou et al., 2011). Absorbance was measured at 510 nm using rutin as standard and results were expressed as rutin equivalents (mg RE/g dw). The CTC was assessed by the 4-dimethylaminocinnamaldehyde (DMACA) method, adapted to 96-well plates (Zou et al., 2011). Absorbance was measured at 640 nm using catechin as standard and results were expressed as mg of catechin equivalents (mg CE/g dw).

2.4.2. Phenolic composition by HPLC

The extracts were dissolved in ultrapure water, or other appropriate solvent, at the concentration of 10 mg/mL and analysed by HPLC–DAD (Agilent 1100 Series LC system, Germany) equipped with vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A) and a diode array detector (G1315B). Data acquisition and instrumental control were performed with LC3D ChemStation software (Rev.A.10.02[1757] version, Agilent Technologies). Analyses were carried out on a mediterranea sea 18 column (15×0.21 cm, 5 µm particle size; Teknokroma, Spain). The mobile phase consisted on a mixture of methanol (solvent A) and 2.5% acetic acid aqueous solution, following a gradient of 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, with 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 (for quantification), 320 and 350 nm. Levels of the different compounds were extrapolated from calibration curves prepared individually for each commercial standard in methanol (apigenin, butylated hydroxytoluene (BHT), caffeic acid, catechin, chlorogenic acid, coumaric acid, epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, flavone, gallic acid, gentisic acid, 4-hydroxybenzaldehyde, m-hydroxybenzoic acid, p-hydroxybenzoic acid, luteolin, oleanolic acid, quercetin, resveratrol, rosmarinic acid, rutin hydrate, salicylic acid, syringic acid, transcinnamic acid, uvaol, vanillic acid and verbascoside) or ultrapure water (luteolin-7-*O*-glucoside), at 1.00 mg/L, and diluted to desired concentrations with ultrapure water.

2.5. Antioxidant activity

2.5.1. Radical scavenging activities (RSA) on DPPH, nitric oxide (NO) and ABTS radicals

The capacity to scavenge the free radicals DPPH, NO and ABTS was evaluated according to Brand-Williams et al. (1995), Baliga et al. (2003) and Re et al. (1999), respectively, adapted to 96-well plates as described by Rodrigues et al. (2015). BHT (1 mg/mL) was used as positive control. Results were calculated as percentage of inhibition, relative to a control containing the extracts' solvent, and expressed as IC_{50} values (half maximal inhibitory concentration, ascertained for extracts with activities higher than 50% at 10 mg/mL).

2.5.2. Metal chelating activities on copper (CCA) and iron (ICA)

The CCA and ICA were determined following Megías et al. (2009), as described by Rodrigues et al. (2015). Change in colour was measured at 632 nm for CCA and at 562 for ICA, using the synthetic metal chelator EDTA as positive control (1 mg/mL). Results were expressed as IC_{50} values (mg/mL).

2.6. Total amino acids

For the analysis of total amino acids samples from leaves were hydrolysed with hydrochloric acid (6M) at 106¨°C for 24h in nitrogen-flushed glass vials and afterwards processed and analysed using the PicoTag method (Waters, USA; Cohen et al., 1988). Amino acid analyses were performed by HPLC in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 × 300 mm), using norleucine as an internal standard. Resulting peaks were analysed with the Breeze software (Waters, USA).

2.7. Minerals

Leaves were analysed for minerals (calcium – Ca, cadmium – Cd, chromium – Cr, copper – Cu, iron – Fe, magnesium – Mg, manganese – Mn, nickel – Ni, lead – Pb and zinc – Zn) by flame atomic absorption spectrometry or flame atomic emission spectrometry (potassium – K and sodium – Na) (novAA® 350 - Analytik Jena, SW-version Aspect LS 1.3.2.0), after acid digestion with nitric acid, filtration and dilution. Working standards of different concentrations were prepared from certified standard solutions; for analytical quality assurance, results were corrected by subtracting a blank from the analysed metal concentrations and samples were analysed in triplicate. Detection limits were: Ca 1.234 mg/L, Cd 0.121 mg/L, Cr 0.596 mg/L, Cu 0.356 mg/L, Fe 0.167 mg/L, Mg 0.031 mg/L, Mn 0.044 mg/L, Ni 0.015 mg/L, Pb 1.226 mg/L, Zn 0.081 mg/L; K and Na detection limits were not determined. Results were expressed as mg/g dw.

2.8. Statistical analysis

Results were expressed as mean ± standard deviation (sd), and experiments were conducted at least in triplicate. Significant differences ($p<0.05$) were assessed by one-way analysis of variance (ANOVA) or Kruskal Wallis one-way analysis of variance on ranks when parametricity of data did not prevail. If significant, the pairwise multiple comparison tests Tukey or Dunn's were applied. Statistical analyses were performed using XLStat2014®. IC_{50} values were computed by curve fitting in GraphPad Prism® version 6.0c.

3. Results and Discussion

3.1. Phytoconstituents of P. coronopus: phenolic composition

Phenolic compounds are ubiquitous in plants but as each plant contains different polyphenolic mixtures, characterization of phenolic groups is often difficult. Hence, fast-screening colorimetric (spectrophotometric) techniques are frequently used for the assessment of the total phenolic content or content of specific phenolic classes (Naczk and Shahidi, 2004). *P. coronopus* extracts had high amounts of total polyphenols, flavonoids and condensed tannins (Table 1). Natural extracts are classified as rich in phenolic compounds when their TPC (expressed as GAE values) is higher than 20 mg/g (Ksouri et al., 2012; Rodrigues et al., 2015) and in this sense all *P. coronopus*

Table 1. Phenolic content[1] (mg/g biomass dw) in *P. coronopus* roots, leaves and flowers extracts: total polyphenols (TPC), total flavonoids (TFC) and condensed tannin (CTC) content. In each column different letters mean significant statistical differences between phenolic contents of the extracts ($p<0.05$).

Organ	Extract	TPC (mg GAE/g)	TFC (mg RE/g)	CTC (mg CE/g)
Roots	Hexane	0.56 ± 0.02^{g}	0.10 ± 0.05^{b}	0.04 ± 0.00^{d}
	Ethyl acetate	1.34 ± 0.05^{fg}	5.38 ± 0.85^{b}	0.19 ± 0.03^{d}
	Methanol	30.6 ± 1.70^{a}	82.2 ± 19.4^{ab}	1.21 ± 0.18^{cd}
	Water	8.78 ± 0.51^{e}	16.5 ± 2.77^{b}	1.32 ± 0.07^{cd}
	Total	**41.3**	**104**	**2.76**
Leaves	Hexane	2.07 ± 0.08^{fg}	12.1 ± 3.20^{b}	1.11 ± 0.14^{cd}
	Ethyl acetate	2.43 ± 0.12^{f}	12.6 ± 2.48^{b}	0.74 ± 0.08^{cd}
	Methanol	28.1 ± 1.07^{b}	146 ± 18.3^{a}	6.17 ± 1.06^{a}
	Water	9.98 ± 1.57^{de}	56.0 ± 15.2^{ab}	3.14 ± 0.35^{b}
	Total	**42.6**	**226**	**11.2**
Flowers	Hexane	1.26 ± 0.07^{fg}	7.73 ± 1.27^{b}	0.48 ± 0.05^{d}
	Ethyl acetate	1.53 ± 0.14^{fg}	1.60 ± 0.56^{b}	0.36 ± 0.06^{d}
	Methanol	15.7 ± 1.41^{c}	34.0 ± 9.41^{b}	3.19 ± 0.26^{b}
	Water	11.0 ± 1.33^{d}	13.4 ± 2.63^{b}	1.96 ± 0.20^{c}
	Total	**29.5**	**56.8**	**6.00**

[1]Data represent the mean ± sd ($n \geq 6$). GAE – Gallic acid equivalents; RE – Rutin equivalents; CE – Catechin equivalents.

organs had high phenolic content. TPC was within the range of values found in literature for *P. coronopus* and for other *Plantago* species (Beara et al., 2009; Janković et al., 2012; Rodrigues et al., 2014). Globally, leaves had the highest content of all phenolic groups, an accumulation pattern possibly linked to the phenolics role in plant physiological survival since phenolics, and flavonoids in particular, can act like UV filters protecting the photosynthetic tissues in the leafs, the largest radiation-exposed area (Harborne and Williams, 2000). In fact, flavonoids were the major constituents of almost all extracts, ranging from 0.10 mg RE/g in the hexane extract of roots to 146 mg RE/g in the methanol extract of leaves. The total flavonoid contents of *P. coronopus*' organs were particularly higher than those found in the literature for different plantains, for example *P. major* and *P. lanceloata* (Beara et al., 2009, 2012b). The observed differences may be related to different extraction procedures and/or analytical methods used, or harvesting time and environmental characteristics that can also influence compounds concentration in plantains (Tamura and Nishibe, 2002). Nonetheless, flavonoids are one of the most characteristic classes of compounds in *Plantago* species (Ksouri et al., 2012). Although polyphenolic content varied considerably amongst the different extracts, the more polar extracts, particularly methanol, had the highest phenolic contents. Solvents' polarity defines the composition of the obtained extracts and methanol is known for its affinity to a broad diversity of bioactive compounds, namely phenolics (Khoddami et al., 2013).

The HLPC-DAD analysis of *P. coronopus* extracts led to the identification of eighteen compounds: ten phenolic acids, seven flavonoids and one other polyphenol (Table 2). From these, only a few were already described in *P. coronopus*, namely caffeic acid, verbascoside, quercetin, luteolin-7-O-glucoside, luteolin and apigenin (Jurišić Grubešić et al., 2013; Janković et al., 2012). Salicylic acid and epicatechin were, to the best of our knowledge, here firstly described in *Plantago* species.

The phenolic profile of *P. coronopus* varied according to the organ and extraction solvent. Flowers had a higher phenolic diversity with 17 out of the 18 compounds identified and two of them exclusively present in this organ: caffeic acid and catechin. Verbascoside was the main phenolic identified in all organs corresponding to 95%, 78% and 85% of the total phenolics in roots, leaves and flowers, respectively. Roots had the highest content of verbascoside, followed by leaves and flowers, which might be explained by the allelopathic properties of that compound on soil phytopathogenic fungi, inhibiting their growth (Egorov et al., 2004). Described as a main compound in plantains, verbascoside levels in this study were higher than those reported by other authors for the aerial parts of *P. coronopus* and five other *Plantago* species (Janković et al., 2012). This major component has known antioxidant, anti-inflammatory, and antifungal properties, along with cytotoxic activity (Egorov et al., 2004; Gálvez et al., 2005; Ksouri et al., 2012).

Luteolin-7-O-glucoside was the second most abundant phenolic identified, with similar concentrations between organs, and its levels were higher than those reported for *P. coronopus* by other authors (Janković et al., 2012). This compound has demonstrated antioxidant and cytotoxic activities (Gálvez et al., 2003, 2005). Ferulic, salicylic and rosmarinic acids followed as major compounds identified, all present in higher amounts in roots. Ferulic acid plays a vital role in providing rigidity to plant cell walls (Kumar and Pruthi, 2014) and has many recognized bioactivities particularly antioxidant, anti-inflammatory, neuroprotective

Table 2. Phenolic profile (mg/g dw) of *P. coronopus* roots, leaves and flowers extracts, analysed by HPLC-DAD.

Classes	RT (min)	Compound (Peak)	Roots				Leaves				Flowers			
			Hex	EtOAc	MeOH	H_2O	Hex	EtOAc	MeOH	H_2O	Hex	EtOAc	MeOH	H_2O
Phenolic acids														
Hydroxybenzoic acids														
	1.58	Gallic acid	0.04	0.21	-	-	-	0.20	-	-	0.05	0.10	0.10	-
	4.72	p-Hydroxybenzoic acid	-	0.31	-	0.16	-	0.45	0.2	0.18	-	-	0.12	0.15
	7.05	Vanillic acid	-	0.21	0.04	-	-	0.26	-	-	-	0.05	0.07	-
	9.78	Syringic acid	0.04	0.05	0.03	-	-	-	-	-	-	-	0.07	-
	12.8	Salicylic acid	-	0.30	1.53	-	0.07	0.42	0.72	-	-	-	1.06	-
Hydroxycinnamic acids														
	8.18	Caffeic acid	-	-	-	-	-	-	-	-	0.02	-	-	-
	12.0	Coumaric acid	0.02	0.11	0.12	0.08	0.02	0.11	0.09	0.07	0.03	0.02	0.09	0.06
	13.3	Ferulic acid	-	0.44	1.62	0.59	0.02	0.31	0.76	0.48	-	-	1.23	0.40
	14.9	Verbascoside	0.09	150	86.6	2.76	0.08	3.67	32.9	4.89	0.27	5.43	45.7	8.9
	17.7	Rosmarinic acid	-	0.58	0.72	-	-	-	0.66	-	-	-	-	-
Flavonoids														
Flavanols														
	5.36	Catechin	-	-	-	-	-	-	-	-	-	-	0.57	-
	10.7	Epicatechin	0.05	0.18	-	-	-	0.32	-	-	0.05	-	0.20	-
Flavonols														
	22.0	Quercetin	-	-	-	0.37	-	-	-	0.43	-	-	-	0.24
Flavones														
	17.3	Luteolin-7-O-glucoside		1.86	1.37	1.54		0.16	1.34	3.62		0.31	1.31	3.45
	22.7	Luteolin		0.16	0.05	0.03			0.04	0.28			0.32	0.14
	25.5	Apigenin	0.08	-	-	-	0.06	-	-	-	0.06	-	-	-
	27.7	Flavone	0.04	-	-	-	0.03	-	-	-	0.02	-	-	-
Other polyphenols														
	5.65	4-Hydroxybenzaldehyde	0.01	0.05	-	-	0.01	0.07	-	-	0.01	0.01	-	-
		Total	**0.37**	**154**	**92.0**	**5.53**	**0.28**	**5.96**	**36.7**	**9.95**	**0.5**	**5.92**	**50.8**	**13.3**
				Roots total		***252***		***Leaves total***		***52.9***		***Flowers total***		***70.6***

RT – retention times; Hex – Hexane; EtOAc – Ethyl acetate; MeOH – Methanol; H_2O – Water.

and anti-carcinogenic (Chao and Lin, 2011). Salicylic acid was here identified for the first time in *Plantago* species. This phenolic was first isolated from willow bark (*Salix spp.*) in pursuit of the active ingredient responsible for the tree's medicinal properties and soon after acetylsalicylic acid (aspirin) was synthesized (Raskin, 1992). Salicylic acid has been implicated in the improvement of stress tolerance in plants, like acclimation to saline stress (Singh and Gautam, 2013), and has anti-inflammatory, antipyretic, analgesic, antiseptic, antifungal and keratolytic properties (Khadem and Marles, 2010). Rosmarinic acid (RA), although already isolated in *P. lagopus* (Fiz et al., 2000), is here firstly quantified in the *genus*. The RA content was within the range of that determined for *Rosmarinus officinalis*, a traditional folk remedy from which the compound was firstly isolated (Moreno et al., 2006; Petersen and Simmonds, 2003). This phenolic has been reported as a phago-deterrent used by plants as a defence against pathogens and herbivores (Petersen and Simmonds, 2003), and has antioxidant, antimicrobial, neuroprotective, anti-inflammatory and antiviral activities (Moreno et al., 2006; Petersen and Simmonds, 2003). These major phenolic compounds identified in *P. coronopus* extracts (verbascoside, luteolin-7-O-glucoside, ferulic, salicylic and RA) can support the main traditional medicinal uses given to this halophyte given their reported bioactivities.

The *p*-hydroxybenzoic and vanillic acids, quercetin and epicatechin were also detected in high levels in all organs (especially in leaves), along with luteolin (higher in flowers), gallic and coumaric acids (higher in roots), coumaric being the only phenolic present in all extracts. The values obtained for the phenolic acids were similar to those found in literature for several other plantain species (Beara et al., 2012a, b; Janković et al., 2012). Those compounds are allelopathic (Seal et al., 2004) (except gallic acid) and have several bioactivities, for example antimutagenic and antitumor (*p*-hydroxybenzoic, gallic and coumaric acids), antimicrobial (*p*-hydroxybenzoic, vanillic and coumaric acids), anti-inflammatory (gallic and coumaric acids) and neuroprotective (coumaric acid) (Khadem and Marles, 2010; Ksouri et al., 2012; Vauzour et al., 2010). As for flavonoids, quercetin and luteolin were abundant in *P. coronopus* (Table 2). These flavonoids are reported to exert a variety of effects, for example neuroprotective and antimicrobial (quercetin, epicatechin), antiproliferative and anti-adipogenic (quercetin) and cytotoxic (luteolin) (Huang et al., 2006; Ksouri et al., 2012; Vauzour et al., 2010; Vuuren, 2008). The minor compounds identified in *P. coronopus* extracts were 4-hydroxybenzaldehyde, apigenin and flavone, present only on the less polar extracts of all organs, along with syringic acid, found in roots and flowers.

3.2. Antioxidant activity

The crude extracts of roots, leaves and flowers were tested for radical scavenging and metal chelating potential using complementary *in vitro* assays, attending at the multifaceted aspects of antioxidants and their reactivity. It is increasingly documented that compounds with RSA effectively prevent oxidative damage and their consumption has become a strategy to address health challenges (Ksouri et al., 2012). Moreover, agents with the ability to chelate metal ions like Fe and Cu, through the formation of inactive complexes, can prevent metal-induced ROS and oxidative stress (Rodrigues et al., 2014, 2015).

The RSA and metal chelating properties were highly variable among *P. coronopus* organs, as shown in Table 3.

Table 3. Antioxidant activity of *P. coronopus* roots, leaves and flowers extracts (IC_{50} values, mg/ml): radical scavenging on DPPH, ABTS and NO radicals, and metal chelating activities on copper (CCA) and iron (ICA).

Organ	Extract	DPPH	ABTS	NO	CCA	ICA
Roots	Hexane	>10	2.43 ± 0.27^{a}	3.66 ± 0.37^{cd}	4.76 ± 0.19^{cd}	1.95 ± 0.13^{c}
	Ethyl acetate	0.47 ± 0.03^{g}	0.57 ± 0.03^{f}	6.71 ± 0.49^{b}	4.28 ± 0.31^{ef}	>10
	Methanol	0.51 ± 0.04^{g}	0.56 ± 0.02^{f}	>10	5.74 ± 0.40^{b}	>10
	Water	0.81 ± 0.09^{ef}	1.79 ± 0.11^{b}	>10	4.07 ± 0.48^{f}	6.29 ± 0.60^{a}
Leaves	Hexane	8.56 ± 1.03^{a}	>10	3.21 ± 0.31^{d}	7.20 ± 0.77^{a}	1.08 ± 0.07^{e}
	Ethyl acetate	1.66 ± 0.23^{c}	1.85 ± 0.17^{b}	10.5 ± 1.18^{a}	>10	>10
	Methanol	1.21 ± 0.06^{d}	1.39 ± 0.15^{d}	>10	7.75 ± 0.90^{a}	>10
	Water	1.88 ± 0.23^{c}	1.58 ± 0.09^{c}	3.83 ± 0.31^{c}	4.61 ± 0.19^{de}	5.43 ± 0.28^{b}
Flowers	Hexane	>10	>10	>10	4.80 ± 0.26^{cd}	1.29 ± 0.09^{d}
	Ethyl acetate	4.33 ± 0.17^{b}	2.55 ± 0.22^{a}	>10	>10	>10
	Methanol	0.88 ± 0.05^{e}	0.82 ± 0.05^{e}	>10	5.50 ± 0.72^{bc}	>10
	Water	0.76 ± 0.05^{f}	1.33 ± 0.02^{d}	3.55 ± 0.55^{cd}	3.03 ± 0.15^{g}	5.77 ± 0.64^{ab}
BHT		0.32 ± 0.02^{h}	0.11 ± 0.01^{g}			
Ascorbic acid				2.34 ± 0.26^{e}		
EDTA					0.13 ± 0.01^{h}	0.07 ± 0.00^{f}

In each column different letters mean significant statistical differences between IC_{50} values of the extracts ($p<0.05$).

Roots had the highest RSA towards DPPH and ABTS radicals, namely in the ethyl acetate and methanol extracts, while the NO radical was best scavenged by the leaves' hexane extract, followed by roots' hexane and flowers' water extracts. The Cu^{2+} and Fe^{2+} chelating capacities of *P. coronopus* extracts were moderate; the water extract from flowers had the highest Cu^{2+} chelating potential, while the hexane extract from leaves displayed the best capacity to chelate Fe^{2+}. Extracts from the leaves of *P. coronopus* had lower RSA activity than those reported for leaves' extracts of different *Plantago* species (Beara et al., 2009, 2012a,b; Gálvez et al., 2005). These differences can be due to the different extracts tested, since parameters like solvent type and extraction method may influence the extracted antioxidant compounds (Khoddami et al., 2013), or due to biological and/or environmental conditions that influence the plants composition and contents of active constituents (Ksouri et al., 2012; Tamura and Nishibe, 2002).

Overall, our results showed variability between *P. coronopus* organs regarding antioxidant activities: root extracts had better radical scavenging activity towards DPPH and ABTS radicals, leaves had high RSA particularly on NO radical and greater iron chelating ability, while flower extracts were best at chelating copper. This organ-related bioactivity has been described for other halophytes, such as *M. edule* and *Limonium algarvense* where it was attributed to different accumulation patterns of secondary metabolites, mainly phenolics that differ between organs (Ksouri et al., 2008; Rodrigues et al., 2015). However, the highest content of all phenolic groups where mainly found on leaves (Table 1). It is commonly found in literature that a high phenolic content correlates with antioxidant activity (Ksouri et al., 2012) but other authors found no such relation (Kähkönen et al., 1999). In fact, colorimetric assays give estimates of total phenolic content (Ignat et al., 2011) but may not necessarily incorporate all the antioxidants present in an extract (Tawaha et al., 2007). Phenolic compounds are known powerful antioxidants (Ksouri et al., 2012; Takao et al., 2015), and the higher radical scavenging or chelating activities of each organ may be explained by the amount and nature of individual phenolic compounds (Table 2). Roots had a great amount of verbascoside, a known antioxidant, particularly in the extracts with the highest RSA, along with higher levels of ferulic, salicylic, rosmarinic, gallic and coumaric acids; leaves had higher levels of luteolin-7-O-glucoside, *p*-hydroxybenzoic acid, vanillic acid, quercetin and epicatechin, and flowers had caffeic acid and catechin exclusively along with greater phenolic diversity, possibly conferring to each of these organs their specific properties.

3.3. Phytoconstituents of P. coronopus: minerals and amino acids in leaves

Several species belonging to the *Plantago* genus, such as *P. major*, *P. lanceolata* and *P. media*, contain interesting levels of essential amino acids and minerals, thus increasing their potential as food and/or as food ingredients (Guil-Guerrero, 2001; Amaglo et al., 2010). In this sense, the edible organs of *P. coronopus*, i.e. the leaves, were analysed for minerals and amino acids, and results are summarized on Tables 4 and 5. *P. coronopus* has high levels of Na (50.0 mg/g dw), is a good source of Ca (14.0 mg/g) surpassed only by Ca-rich leaves like arugula or watercress (19.3 and 24.5 mg/g dw; USDA 2015), and has also good levels of Fe (0.41 mg/g dw) and Mg (6.34 mg/g), comparable to dark green vegetables like spinach (USDA 2015). The levels of Ca, Mg and K are higher than those reported for other halophytes such as *Salicornia* species (Díaz et al., 2013; Essaidi et al., 2013), whereas Mn and Zn were found in small quantities just like in other vegetables (Díaz et al., 2013; Essaidi et al., 2013). When compared to other *Plantago* species (*P. major*, *P. media*, *P. lanceolata*), *P. coronopus* has higher Na, Ca, Mg, Fe and Zn levels (Guil-Guerrero, 2001). Moreover, potentially toxic minerals like Cu, Cr, Ni, Cd and Pb were not detected in this study.

Regarding the amino acid (AA) profile of *P. coronopus* leaves (Table 5) arginine was the major essential AA (10.2 mg/g dw) followed by leucine (5.57 mg/g dw) and threonine (5.22 mg/g dw), while tryptophan (0.05 mg/g dw) and histidine (1.40 mg/g dw) were the minor AA. Arginine is a conditionally essential AA: it is required in higher amounts than can be produced during growth development, and dietary deficiency can result in metabolic, neurological or reproductive dysfunction (Wu, 2009). Our results indicate that *Plantago*'s leaves are a good arginine source for young/developing mammals, akin to other vegetables like lettuce or asparagus (USDA, 2015). As for the other AA, threonine (45.1 mg/g protein dw) along with the sum of phenylalanine and tyrosine (80.9 mg/g protein dw) exceed the FAO/WHO (WHO, 1973) reference values for AA composition, making this plantain's leaves a good source for these AA. Tryptophan is the most limiting amino acid since it is in greater deficit, but the remaining amino acid levels in *P. coronopus* represent between 60% and 70%

Table 4. Mineral content[1] (mg/g dw) of *P. coronopus* leaves.

Classification	Mineral	Symbol	Content
Essential elements			
	Sodium	**Na**	50.0 ± 2.45
	Calcium	**Ca**	14.0 ± 0.55
	Potassium	**K**	8.02 ± 0.12
	Magnesium	**Mg**	6.34 ± 0.28
	Iron	**Fe**	0.41 ± 0.02
	Manganese	**Mn**	0.01 ± 0.00
	Zinc	**Zn**	0.05 ± 0.00
	Copper	**Cu**	BDL
	Chromium	**Cr**	BDL
	Nickel	**Ni**	BDL
Non-essential elements			
	Lead	**Pb**	BDL
	Cadmium	**Cd**	BDL

[1]Data represent the mean ± sd (n = 3); BDL – below detection limit.

Table 5. Amino acid profile[1] (total AA, mg/g leaves dw) of *P. coronopus* leaves (mg/g protein dw).

Amino acids	Total AA (mg/g leaves dw)
Essential AA	
Isoleucine	3.34 ± 0.14
Histidine	1.40 ± 0.03
Leucine	5.57 ± 0.31
Lysine	4.17 ± 0.07
Methionine	2.01 ± 0.05
Phenylalanine	4.83 ± 0.03
Threonine	5.22 ± 0.08
Tryptophan	0.05 ± 0.00
Valine	3.51 ± 0.12
Arginine*	10.2 ± 0.63
Total essential AA	**40.3 ± 0.25**
Non-essential AA	
Alanine	5.33 ± 0.27
Aspartic acid	0.86 ± 0.07
Asp + Asparagine	8.41 ± 0.05
Cysteine	0.42 ± 0.02
Glutamic acid	0.77 ± 0.01
Glut + Glutamine	8.66 ± 0.42
Glutamine	0.69 ± 0.02
Glycine	5.21 ± 0.07
Ornithine	0.07 ± 0.00
Proline	4.53 ± 0.03
Serine	6.02 ± 0.01
Taurine	0.42 ± 0.02
Tyrosine	4.52 ± 0.15
β-Alanine	0.40 ± 0.01
γ-Amino-n-butyric acid	0.93 ± 0.04
Total non-essential AA	**47.2 ± 0.43**

[1]Data represent the mean ± sd (n = 2). *Conditionally essential: required in greater amounts than can be produced in growth development.

of the FAO/WHO (WHO, 1973) reference values. As for non-essential AA, although synthesized by the body, some can be conditionally essential in neonates (e.g. glutamine, taurine), under stress conditions (e.g. glutamine), or for carnivores and some fish (taurine) (Wu, 2009). In such cases *P. coronopus* leaves can be an additional source of these AA. To our knowledge, this is the first report of the presence of AA like ornithine, taurine, β-alanine and γ-amino-n-butyric acid in *Plantago* species and the first amino acid profile of *P. coronopus*.

4. Conclusions

Our results indicate that the edible halophyte *P. coronopus* has a high polyphenolic content and good antioxidant activity in its different anatomical organs. Moreover, its leaves are valuable source of minerals and amino acids. The phenolic richness known for a wide range of biological activities can explain the main traditional uses given to this plantain and, altogether, these results emphasize the potential of *P. coronopus* as a source of bioactive molecules, especially useful for the prevention of oxidative stress-related diseases, leading to the discovery of new food products.

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CHAPTER 3

HEALTH PROMOTING POTENTIAL OF HERBAL TEAS AND TINCTURES FROM *ARTEMISIA CAMPESTRIS* SUBSP. *MARITIMA*: FROM TRADITIONAL REMEDIES TO PROSPECTIVE PRODUCTS

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Health promoting potential of herbal teas and tinctures from *Artemisia campestris* subsp. *maritima*: from traditional remedies to prospective products

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This work explored the biotechnological potential of the medicinal halophyte *Artemisia campestris* subsp. *maritima* (dune wormwood) as a source of health promoting commodities. For that purpose, infusions, decoctions and tinctures were prepared from roots and aerial-organs and evaluated for *in vitro* antioxidant, anti-diabetic and tyrosinase-inhibitory potential, and also for polyphenolic and mineral contents and toxicity. The dune wormwood extracts had high polyphenolic content and several phenolics were identified by ultra-high performance liquid chromatography–photodiode array–mass-spectrometry (UHPLC-PDA-MS). The main compounds were quinic, chlorogenic and caffeic acids, coumarin sulfates and dicaffeoylquinic acids; several of the identified phytoconstituents are here firstly reported in this *A. campestris* subspecies. Results obtained with this plant's extracts point to nutritional applications as mineral supplementary source, safe for human consumption, as suggested by the moderate to low toxicity of the extracts towards mammalian cell lines. The dune wormwood extracts had in general high antioxidant activity and also the capacity to inhibit α-glucosidase and tyrosinase. In summary, dune wormwood extracts are a significant source of polyphenolic and mineral constituents, antioxidants and α-glucosidase and tyrosinase inhibitors, and thus, relevant for different commercial segments like the pharmaceutical, cosmetic and/or food industries.

Medicinal plants are increasingly explored by the food industry for their health-promoting benefits either as readily available for herbal teas (e.g. *Matricaria chamomilla* [chamomile], *Cymbopogon citratus* [lemongrass]) or as sources of additives for functional foods and drinks (e.g. *Aloe vera* [aloe], *Aspalathus linearis* [rooibos])[1,2]. Yet, medicinal halophytes remain largely unexplored and underutilized despite their outstanding potential as a reservoir of bioactive compounds and innovative health promoting products[3]. Recently, different scientific efforts have unveiled some of these halophytes' prospective commercial uses namely as food (e.g. *Arthrocnemum macrostachyum*[4]), herbal functional beverages (e.g. *Helichrysum italicum* subsp. *picardii*[5], *Crithmum maritimum*[6], *Limonium algarvense*[7]), or as raw material for pharmaceutical and other related industries (e.g. *Lithrum salicaria*[8], *Polygonum maritimum*[9]).

Halophytes live and thrive in saline biotopes characterized by highly fluctuating abiotic constraints. To deal with such unfavourable conditions these salt-tolerant plants developed adaptive responses including the synthesis of highly bioactive molecules with potent antioxidant capacity, such as phenolic compounds, terpenoids and vitamins, to counteract reactive oxygen species (ROS) production and accumulation, inhibit oxidative chain-reactions and protect cellular structures[3]. These natural antioxidants usually display strong biological activities, like radical-scavenging, metal-chelating and enzyme-inhibiting abilities, leading to beneficial therapeutic

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Organ	Extract	Yield	TPC[1]	TFC[2]	CTC[3]	HAD[4]	Flavonols[5]	Anthocyanins[6]
Roots	Infusion	100.9	115 ± 5.03^{b}	25.0 ± 0.85^{c}	<LQ	86.0 ± 0.64^{bc}	59.5 ± 2.35^{b}	1.90 ± 0.26^{b}
	Decoction	142.0	114 ± 3.72^{b}	26.3 ± 1.56^{c}	<LQ	86.7 ± 0.98^{bc}	57.8 ± 2.50^{b}	2.44 ± 1.15^{ab}
	Tincture	12.4	118 ± 5.23^{b}	18.7 ± 0.98^{d}	<LQ	85.0 ± 0.78^{c}	66.2 ± 1.63^{a}	3.36 ± 0.34^{a}
Aerial-organs	Infusion	205.3	121 ± 6.20^{b}	35.5 ± 0.69^{b}	<LQ	89.2 ± 1.66^{a}	54.1 ± 1.62^{c}	1.96 ± 0.51^{b}
	Decoction	208.5	119 ± 6.16^{b}	34.4 ± 0.92^{b}	<LQ	88.4 ± 1.96^{ab}	51.9 ± 1.46^{c}	1.94 ± 0.63^{b}
	Tincture	20.1	134 ± 11.9^{a}	40.8 ± 1.80^{a}	<LQ	89.4 ± 1.83^{a}	60.9 ± 0.94^{b}	3.46 ± 0.53^{a}

Table 1. Phenolic contents (mg/g dry weight, DW) of infusions, decoctions and tinctures from *Artemisia campestris* subsp. *maritima* organs and respective yields (infusion and decoctions: mg extract/200 mL, tinctures: mg extract/mL). Data represent the mean ± SD ($n \geq 6$). In each column, different letters mean significant differences ($p < 0.05$). LQ (limit of quantification) CTC = 0.78 mg CE/g DW. [1]TPC: total polyphenol content, mg GAE/g DW, GAE: gallic acid equivalents. [2]TFC total flavonoid content; mg QE/g DW, QE: quercetin equivalents. [3]CTC: condensed tannin content, mg CE/g DW, CE: catechin equivalents. [4]HAD hydroxycinnamic acid derivatives, mg CAE/g DW, CAE: caffeic acid equivalent. [5]mg QE/g DW, QE: quercetin equivalents. [6]mg CCE/g DW, CCE: cyanidin chloride equivalents.

properties, which can help explain the use of some halophytes in traditional medicine and as dietary plants[3,10]. For example, the aromatic *Crithmum maritimum* is used in folk medicine as diuretic, antiscorbutic, digestive or anti-inflammatory, and is traditionally consumed as condiment, pickle, and in salads[11]. Another aromatic halophyte, *Helichrysum italicum* subsp. *picardii*, is often used as a spice and has folk therapeutic uses such as anti-inflammatory, analgesic or anti-microbial[12]. Besides their traditional use as food and folk remedies, halophytes can be produced in otherwise uncultivable saline soils and marine-influenced environments and serve as alternative cash crops in saline agriculture. In fact, these plants could be explored for diverse commercial segments, from human and animal nutrition to pharmaceutical and cosmetic industries[13,14].

Artemisia campestris L. subsp. *maritima* Arcangeli (Asteraceae), commonly named dune wormwood ("madorneira" or "erva-lombrigueira" in Portugal), is an aromatic and medicinal halophytic shrub common in coastal sand dunes throughout the temperate European Atlantic coast[15,16]. Usually consumed as herbal tea made from stems and leaves, it is described as a remedy to treat gastric disorders, hypertension and rheumatics, being also used for its anthelmintic and abortifacient properties[15]. The species, *A. campestris*, has additional ethnomedicinal uses described such as anti-diabetic, anti-inflammatory and antipyretic[16]. Although several studies have already profiled the phytochemical content and bioactivities of *A. campestris* (revised in Dib *et al.*[16]), only few reports focused on the subspecies *A. campestris* subsp. *maritima*. Research on this particular plant reports compounds like phenolic acids, flavonoids, coumarins, sesquiterpenes and acetophenone derivatives, determined on organic extracts[17–20], and describes the antioxidant and anti-microbial activities of methanolic extracts[20].

In folk medicine, water (infusions and decoctions) and hydro-alcoholic (tinctures) extracts are commonly used to convey the plants' healing properties[21]. Considering the potential health benefits of such botanical extracts, medicinal plants can offer a wide range of bioactive components (e.g. polyphenols) and can be explored as raw material for herbal beverages, foods products or constituents in health promoting commodities[1]. In fact, natural products are currently in high demand and substances with anti-ageing or beauty-enhancement properties (e.g. skin whitening) are on top of consumers list of interest[1]. Other sought beneficial outcomes include management of diabetes mellitus and improvement of cognitive functions, associated with the intake of antioxidants[22]. Biochemical studies on medicinal plants can therefore be extremely useful to identify new sources of relevant products for pharmaceutical, cosmetic and/or food industries, and many *Artemisia* species already find extensive uses as food additives and in perfumery[23]. In this sense, *Artemisia campestris* subsp. *maritima* could be a potential reservoir of bioactive compounds, representing a commercial underexplored opportunity. Therefore, this work's goal was to explore the dune wormwood's biotechnological potential as source of bioactive phytochemicals. For that purpose, infusions, decoctions and tinctures were prepared from above and below-ground organs of *A. campestris* subsp. *maritima* and assessed for polyphenolic and mineral contents, and *in vitro* antioxidant, anti-diabetic and tyrosinase-inhibition potential. A preliminary *in vitro* toxicological assessment was also carried out using mammalian cells. To the best of our knowledge, this is the first time that such an attempt is made with this plant.

Results and Discussion

Phytochemical profile. The polyphenolic content of the extracts was firstly assessed in terms of their total contents of phenolics (TPC), flavonoids (TFC), condensed tannins (CTC), hydroxycinnamic acid derivatives (HAD), flavonols and anthocyanins (Table 1). Phenolic compounds are some of plants most widely occurring secondary metabolites[24]. Although there is no instituted classification in terms of high/low values of total phenolics, some authors state that natural extracts can be considered rich in phenolic compounds when their TPC is higher than 20 mg GAE/g DW[8,25,26]. In this sense, all of *A. campestris* subsp. *maritima* extracts have high phenolics content considering that TPC was between 114 and 134 mg GAE/g DW, with the highest value determined in aerial-organs' tincture. This extract also had the highest flavonoid content (40.8 mg RE/g DW), higher HAD together with aerial-organs' infusion and decoction (89.4–88.4 mg CAE/g DW), and higher anthocyanins along with roots' tincture (3.46 and 3.36 mg CCE/g DW). Flavonols, on the other hand, were highest in roots' tincture (66.2 mg QE/g DW). As for tannins content, it was not found in the dune wormwood samples (below the limit of quantification, which was 0.78 mg/g DW). Working with the same sub-species, Megdiche-Ksouri *et al.*[20] reported

[a]Peak n°	Compound (Peak)	[b]RT (min)	Roots			Aerial-organs		
			Infusion	Decoction	Tincture	Infusion	Decoction	Tincture
	Quinic acid	1.52	13.00	14.00	15.00	24.00	24.00	24.00
	Protocatechuic acid	7.17	0.100	0.090	0.110	0.420	0.430	0.270
	p-Hydroxybenzoic acid	9.55	0.015	≤0.021	0.020	0.087	0.095	0.116
6	Chlorogenic acid	9.72	8.400	9.000	10.00	11.00	10.00	16.00
	4-Hydroxybenzaldehyde	10.27	0.007	0.010	0.017	0.009	0.009	0.018
	Syringic acid	10.32	0.049	≤0.062	0.081	≤0.048	≤0.066	0.047
10	Caffeic acid	10.56	0.920	0.970	0.630	0.920	1.000	1.630
18	Rutin	12.73	0.024	0.021	0.038	0.700	0.740	1.300
	Cynaroside	12.82	≤0.019	≤0.029	≤0.017	0.029	0.034	0.044
19	Coumaric acid	12.93	0.075	0.064	0.100	0.170	0.190	0.330
21	Ferulic acid	13.04	0.070	0.064	0.078	0.034	0.032	0.055
22	Isoquercitrin	13.29	0.021	0.029	0.024	0.120	0.130	0.200
24	Taxifolin	13.53	≤0.047	≤0.071	≤0.042	0.066	≤0.076	0.092
29	Salicylic acid	14.55	0.062	0.049	0.092	0.120	0.120	0.190
41	Luteolin	16.97	0.021	≤0.028	0.022	0.190	0.200	0.470
	Quercetin	17.15	≤0.005	≤0.007	≤0.004	0.052	0.080	0.061
	Naringenin	17.49	≤0.049	≤0.073	≤0.044	≤0.057	≤0.078	0.053
	Apigenin	18.50	≤0.005	≤0.007	≤0.004	0.016	0.016	0.034
	Isorhamnetin	18.57	≤0.048	≤0.073	0.044	0.160	0.200	0.250
	Kaempferol	18.85	0.020	0.030	0.018	0.026	0.036	0.024
		TOTAL	**22.78**	**24.33**	**26.27**	**38.12**	**37.31**	**45.18**

Table 2. Concentrations of compounds in infusions, decoctions and tinctures from *Artemisia campestris* subsp. *maritima* organs (mg/g DW), calculated with reference standards using LC-amMS. Quantitation limits are presented as ≤ LOQs (µg/mg DW). [a]Corresponding peak number in the chromatograms on Fig. 1. [b]RT – retention times.

similar total phenolics (159 mg GAE/g DW) but higher flavonoid (175 mg CE/g DW) and tannin (8.7 mg CE/g DW) contents in methanolic extracts from shoots. These differences could be ascribed not only to the different solvent and extraction procedure, which several studies have showed to greatly influence results, but also to the different analytical methods used[13]. In similar aqueous and hydro-alcoholic extracts from the aerial parts of the species *A. campestris*, other authors determined different level of TPC and TFC, either higher, similar or lower than those presently found[27–33]. These discrepant phytochemical contents may be explained by species-specific factors, harvesting time and/or environmental characteristics, since these variables affect the biosynthesis of secondary metabolites in plants[3,13]. Nevertheless, authors generally consider *A. campestris* rich in phenolic compounds[16,30].

To further explore the phytochemical profile of infusions, decoctions and tinctures from *A. campestris* subsp. *maritima* a generic LC-PDA-MS (liquid chromatography – photodiode array – mass spectrometry) method for moderately polar phytochemicals was employed. The analytical methodology was adapted from De Paepe *et al.*[34], previously validated by those authors for quantitation of phenolic constituents in apple cultivars, and is fully detailed in Pereira *et al.*[5] including performance characteristics, quantification procedures and compound tentative identification specifics. The aim was to (tentatively) identify phytochemical constituents in the dune wormwood extracts, getting an estimate of their concentrations and/or relative abundances when no reference standards were available. The phenolics and respective concentrations are presented in Table 2. As some standards can be expensive or not available, tentative identification of other compounds was accomplished based on available chromatographic and spectral information (Table 3). To get clean product ion spectra of the detected analytes, data dependent fragmentation was used. Product ions are substructures of precursor ions (ions of a particular mass over charge-range [*m/z*-range]), formed during fragmentation: structures were assigned to unknown peaks when both the *m/z*-values and molecular formulae/structures of the precursor and product ions were in agreement. Further information for de-replication was obtained from PDA spectra, in-house and commercial compound databases (PubChem[35], Dictionary of Natural Products[36], ChemSpider[37]) and peer reviewed publications (a more detailed explanation is given in Pereira *et al.*[5]). MS and diagnostic chromatographic data used for compound identification plus literature used for confirmation of compound identity can be found in Table S2 (supplementary material). It is important to mention that during LC-MS analysis different compounds can have different ionization efficiencies and so no absolute quantitative comparison can be made, although relative abundances per compound in-between samples can be calculated (based on the area of their most abundant ion). In this sense, the "maximum area detected" provides semi-quantitative information of compound abundance. Table 3 shows the relative abundances of these tentatively identified constituents. To visualize the extracts' main detected compounds, the UV-chromatograms at combined wavelengths (280–330 nm, the absorption maxima of phenolics) are represented in Fig. 1, despite not showing all the constituents identified (compounds with no assigned peaks had low abundances or possibly their peaks overlapped).

[a]Peak n°	Tentative ID	[b]RT (min)	Roots			Aerial-organs			Maximum area detected
			Infusion	Decoction	Tincture	Infusion	Decoction	Tincture	
1	Chlorogenic acid isomer (isochlorogenic acid A, B or C)	7.77	91	100	62	91	100	88	410 976 815
2	Hydroxybenzoic acid isomer (2,3-Dihydroxybenzaldehyde)	8	99	100	98	66	77	79	169 361 809
3	Hexoside of scopoletin (scopolin)	8.48	30	28	43	73	66	100	185 080 059
4	Hexoside of coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin)	9.02	43	46	65	69	64	100	403 174 862
5	Chlorogenic acid isomer (isochlorogenic acid A, B or C)	9.33	48	50	49	55	56	100	1 097 618 207
	Aesculetin	9.61	19	19	44	43	47	100	68 226 925
7	Chlorogenic acid isomer (isochlorogenic acid A, B or C)	9.88	65	61	68	68	65	100	108 537 786
8	Fraxetin	10.19	100	93	82	60	62	87	82 132 702
9	Coumaric acid hexoside isomer	10.32	4	3	5	63	64	100	180 996 161
11	Coumaric acid hexoside isomer	10.76	6	4	7	69	70	100	102 579 661
12	Coumarin sulfate with 2 methoxy moieties (iso-fraxidin or fraxidin)	11.54	71	68	100	55	55	69	9 544 319 167
13	Coumarin sulfate (fraxetin-*O*-sulfate isomer)	11.65	50	53	31	82	86	100	4 395 956 598
	Not identified ($C_{12}H_{18}O_7S$)	11.74	6	4	9	62	63	100	1 434 495 637
14	Coumarin sulfate (fraxetin-*O*-sulfate isomer)	11.78	75	73	100	34	35	41	23 258 593 384
15	Scopoletin	12.03	68	62	100	46	49	72	231 549 513
16	Coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin)	12.11	93	90	95	64	64	100	256 857 167
17	Coumarin sulfate (scopoletin-*O*-sulfate isomer)	12.19	71	67	100	31	32	41	17 799 164 012
20	Fraxidin-caffeoyl-hexoside	13.03	32	30	45	55	55	100	91 320 194
23	Methoxy-cinnamic acid	13.53	46	52	100	30	34	55	22 190 655
25	Dicaffeoylquinic acid	13.65	92	100	91	59	62	69	1 324 940 207
26	Dicaffeoylquinic acid	14.06	75	77	100	68	64	93	1 473 103 666
27	Dicaffeoylquinic acid methyl ester	14.35	71	62	100	53	47	95	11 010 820
28	Dicaffeoylquinic acid	14.45	63	66	64	79	75	100	2 500 658 869
30	Dicaffeoylquinic acid methyl ester	14.6	34	34	46	67	76	100	24 968 283
31	Dicaffeoylquinic acid	14.79	44	44	55	52	48	100	190 893 063
32	Dicaffeoylquinic acid methyl ester	15.03	20	19	23	69	70	100	35 407 749
33	Dicaffeoylquinic acid methyl ester	15.37	9	8	11	52	47	100	136 311 796
34	Caffeic acid coupled to $C_{11}H_{12}O_6$	15.89	6	3	3	97	100	100	206 701 996
35	Flavonoid	15.99	3	2	3	59	60	100	207 351 776
36	Ethoxy or dimethoxycinnamic acid	16.1	0	0	43	0	0	100	3 704 845 930
37	Tricaffeoylquinic acid	16.13	14	13	23	11	11	100	793 875 076
38	Dimethoxyflavonoid (axillarin)	16.41	2	1	3	48	51	100	635 257 416
39	Methoxyflavonoid (tamarixetin, rhamnetin, eupafolin, quercetin-3-methylether)	16.72	2	2	3	54	55	100	806 869 736
40	Methoxyflavonoid (laricitrin or mearnsetin)	16.8	0	1	1	61	100	86	89 733 523
	Trimethoxyflavonoid	17.49	2	1	2	48	49	100	40 757 681
42	Dimethoxyflavonoid (cirsiliol)	17.97	2	1	3	53	56	100	714 900 053
43	Trimethoxyflavonoid	18.13	2	1	3	53	55	100	171 818 097
44	Methoxyflavonoid (hispidulin)	18.26	2	1	3	53	56	100	738 797 835
45	Trimethoxyflavonoid (cirsilineol or eupatorin)	18.89	1	1	2	44	45	100	53 729 586
46	Tetramethoxyflavonoid	19.25	2	1	3	49	48	100	138 813 684
	Trimethoxyflavonoid (cirsilineol or eupatorin)	19.48	3	2	4	49	49	100	40 802 811
	Dimethoxyflavonoid (cirsimaritin)	20.07	2	0	2	41	42	100	17 338 075
	Linderoflavone B	21.2	NF	NF	NF	37	47	100	591 906

Table 3. Average relative abundances (peak area/mg DW, %) of the tentatively identified compounds in extracts from *Artemisia campestris* subsp. *maritima* organs, analysed by LC-PDA-amMS. NF – not found. [a]Corresponding peak number in the chromatograms on Fig. 1. [b]RT – retention times.

According to Table 2, the dune wormwood aerial-organs' extracts had greater diversity and higher levels of practically all phenolics found. Aerial-organs' tincture in particular had higher concentrations of most of the determined compounds adding up to a total of 45 µg/mg DW. From this total, quinic acid amounts to half (24 µg/mg DW),

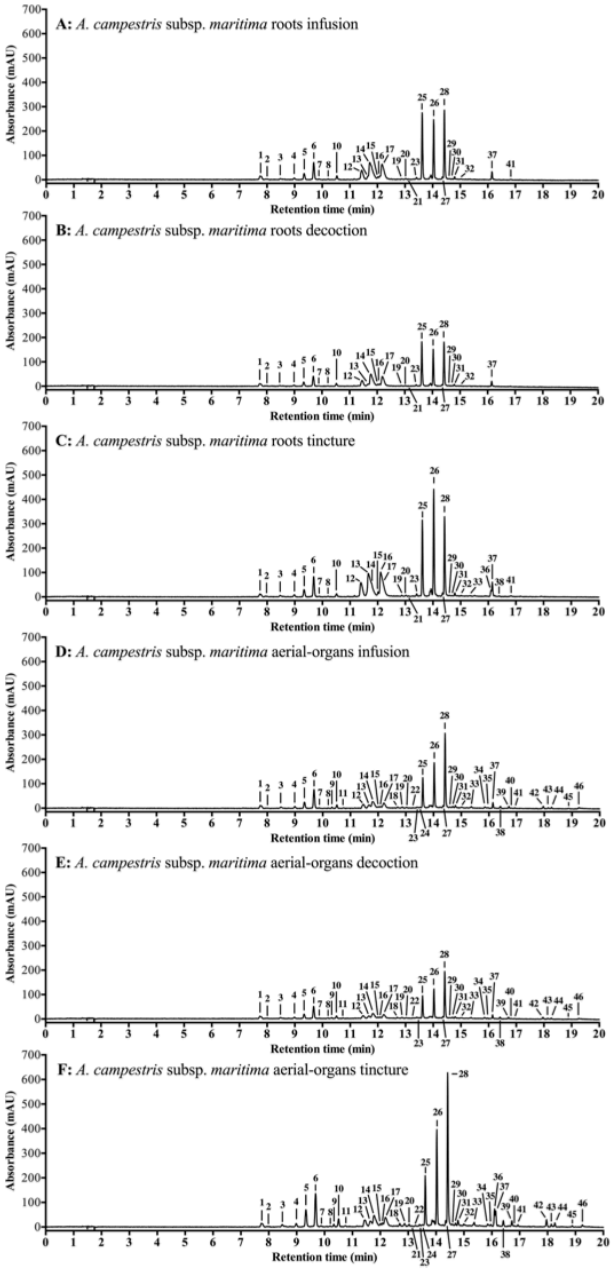

Figure 1. PDA chromatograms (280 + 330 nm) of the extracts from *A. campestris subsp. maritima* roots (**A**) infusion, (**B**) decoction, (**C**) tincture) and aerial-organs (**D**) infusion, (**E**): decoction, (**F**) tincture). Peak numbers refer to compounds listed in Tables 2 and 3.

followed by chlorogenic (16 µg/mg DW) and caffeic (1.6 µg/mg DW) acids. In fact, these phenolic acids were the main constituents determined in all extracts particularly quinic (roots: 13–15 µg/mg DW, aerial-organs: 24 µg/mg DW) and chlorogenic (roots: 8.4–10 µg/mg DW, aerial-organs: 10–16 µg/mg DW) acids, both higher in tinctures. Rutin was also preferentially detected in aerial-organs aqueous and hydro-alcoholic samples (0.7–1.3 µg/mg DW), followed by protocatechuic acid (0.27–0.43 µg/mg DW), luteolin (0.19–0.47 µg/mg DW) and coumaric acid (0.17–0.33 µg/mg DW), along with isoquercitrin, isorhamnetin and salicylic acid (~0.1–0.2 µg/mg DW). In roots' extracts, protocatechuic acid in all extracts (0.09–0.11 µg/mg dw), and coumaric and salicylic acids in

	Mineral	Roots			Aerial-organs		
		Infusion	Decoction	Tincture	Infusion	Decoction	Tincture
	Macro-elements (mg/g)						
Essential elements	Na	17.5 ± 1.79^{bc}	19.4 ± 1.43^{b}	9.10 ± 0.65^{d}	32.6 ± 1.79^{a}	32.4 ± 1.19^{a}	12.3 ± 1.88^{cd}
	Ca	2.91 ± 0.02^{b}	2.99 ± 0.11^{b}	0.09 ± 0.01^{c}	4.53 ± 0.05^{a}	4.29 ± 0.19^{a}	0.15 ± 0.01^{c}
	K	11.0 ± 1.55^{b}	11.4 ± 1.63^{ab}	3.32 ± 0.33^{c}	15.6 ± 1.49^{a}	14.5 ± 0.20^{ab}	3.67 ± 0.37^{c}
	Mg	1.67 ± 0.12^{a}	1.65 ± 0.06^{a}	0.52 ± 0.08^{c}	1.32 ± 0.05^{b}	1.16 ± 0.06^{b}	0.39 ± 0.03^{c}
	Micro and trace-elements (µg/g)						
	Fe	1059 ± 105^{a}	926 ± 57.1^{a}	<LOQ	630 ± 98.7^{b}	626 ± 22.5^{b}	22.0 ± 0.82^{c}
	Mn	76.8 ± 2.72^{ab}	70.7 ± 3.15^{b}	3.31 ± 1.97^{c}	87.9 ± 6.77^{a}	79.9 ± 3.59^{ab}	3.75 ± 0.31^{c}
	Zn	16.2 ± 1.88^{a}	18.3 ± 0.55^{a}	<LOQ	17.4 ± 2.88^{a}	18.0 ± 2.33^{a}	2.30 ± 0.85^{b}
	Cu	27.2 ± 0.93^{ab}	31.5 ± 5.08^{a}	6.70 ± 0.00^{c}	14.6 ± 0.44^{bc}	13.2 ± 1.42^{bc}	1.81 ± 0.90^{c}
	Cr	0.54 ± 0.01^{a}	0.79 ± 0.03^{a}	0.32 ± 0.00^{a}	0.79 ± 0.06^{a}	0.78 ± 0.05^{a}	0.11 ± 0.00^{a}
	Ni	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Non-essential elements	Pb	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Cd	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 4. Mineral content (mg or µg/g DW) in extracts of infusions, decoctions and tinctures from *Artemisia campestris* subsp. *maritima* organs. Data represent the mean ± SD ($n = 3$). In each row different letters mean significant differences ($p < 0.05$). LOQs: Fe: 0.48 µg/g, Zn: 0.88 µg/g, Ni: 0.31 µg/g, Pb: 0.71 µg/g, Cd: 0.40 µg/g of extract DW.

tincture (0.1 and 0.09 µg/mg dw, respectively) were also found in higher levels, although in comparatively lower concentrations than in the aerial-organ's extracts. In Table 3 and Fig. 1 it is also possible to observe the higher compound diversity in extracts from aerial-organs, especially tinctures. However, relative abundance of some major constituents such as coumarin sulfates (peaks 12, 14 and 17) and dicaffeoylquinic acids (peaks 25 and 26) was higher in roots' extracts, particularly tincture. Aerial-organs' extracts had higher amounts of another coumarin sulfate (peak 13) and dicaffeoylquinic acid (peak 28), along with a chlorogenic acid isomer (peak 5) and an ethoxy/dimetoxycinnamic acid (peak 36). Again, it should be stated that Table 3 provides relative quantitative measures of abundance, not to be interpreted as absolute quantitative comparison. Overall, tinctures of both organs showed higher abundance and diversity of constituents comparatively to aqueous extracts and, between organs, extracts from aerial-organs had greater variety of phenolics, generally in higher levels. To the best of our knowledge, this is the first report comparing anatomical organs in this *Artemisia* species. Megdiche-Ksouri *et al.*[20] also report a wide assortment of phytochemicals in dune wormwood's shoots, several of them also presently determined, but studies detailing compound abundance in *A. campestris* extracts other than essential oils are extremely scarce. In fact, only Jahid *et al.*[33] reports levels of phenolics in leaves' hydro-alcoholic extracts with the main components catechin and vanillic acid (>20 mg/g DW), not being found in the current study, syringic (6 mg/g DW) and coumaric (0.9 mg/g DW) acids, presently determined at lower concentrations (0.05–0.08 mg/g DW and 0.06–0.33 mg/g DW, respectively), and caffeic acid (0.2 mg/g DW), being one of the current main constituents particularly in aerial-organs' tincture (1.6 mg/g dw). These authors[33] also consider that compound nature and abundance are related to environmental conditions, a well-established notion when comparing intra-species phytochemical content[3,13,38,39].

Nevertheless, and although differing considerably between subspecies[38], the phenolic profiles of *A. campestris* compiled in literature are generally in agreement with that reported here and include compounds like phenolic acids such as caffeic, chlorogenic, isochlorogenic and other dicaffeoylquinic acids, flavonoids such as apigenin, rutin, luteolin, kaempferol and quercetin, or hydroxycoumarins like aesculetin and scopoletin[16,19,20,40–42]. In fact, from the wide variety of phenolic constituents (tentatively) identified in *A. campestris* subsp. *maritima* extracts (Tables 2 and 3), most if not all were already described in the *Artemisia* genus. However, for the species *A. campestris* no reports were found detailing quinic, protocatechuic, *p*-hydroxybenzoic and salicylic acids, 4-hydroxybenzaldehyde, cynaroside, isoquercitrin and taxifolin (although its derivatives are described), which are, to the best of our knowledge, here described for the first time in the species. Moreover, chlorogenic, syringic, caffeic, coumaric and ferulic acids, luteolin, apigenin and kaempferol were not found reported in the literature for the subspecies under study (although derivatives for the three later are reported) and are therefore here firstly described in *A. campestris* subsp. *maritima*.

Mineral composition. Aqueous extracts like herbal teas can be considered an added source of minerals for the human diet[2,6]. In this context, the presence of these essential nutrients in the dune wormwood's extracts could be of added value for their potential use as food products or in herbal beverages. Hence, *A. campestris* subsp. *maritima* extracts were analysed for mineral content and Table 4 summarizes the results. The most abundant element was Na (9.10–32.6 mg/g DW), followed by K (3.32–15.6 mg/g DW) and Ca (0.09–4.53 mg/g DW), all in higher levels in aerial-organs aqueous extracts. Magnesium (Mg: 0.39–1.67 mg/g DW) and Fe (22–1059 µg/g DW) were also relatively abundant but with higher levels in roots aqueous extract. Mn and Zn were determined in lower concentrations (Mn: 3.31–87.9 µg/g DW; and Zn: 2.30–18.3 µg/g DW). Mn was more abundant in aerial-organs aqueous samples and Zn had similar levels on aqueous extracts of both above and below-ground

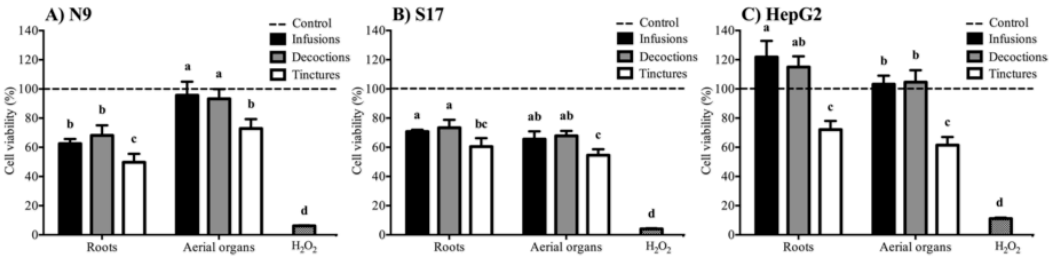

Figure 2. Toxicity of infusions, decoctions and tinctures (100 µg/mL extract dw) from *Artemisia campestris* subsp. *maritima* organs on mammalian cell lines: (**A**) N9, (**B**) S17 and (**C**) HepG2. Cells treated only with cell culture medium were used as controls; H_2O_2 was used as positive control for cell toxicity. Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each graph, different letters mean significant differences ($p < 0.05$).

organs. Moreover, tinctures had consistently lower mineral content showing that water extracts are better at extracting these nutrients from the plant. In fact, herbal teas are usually considered good sources of many elements such as Na, Ca, K, Mg, Fe, Mn or Zn[2]. Considering the adult daily dietary reference mineral intakes (Na: 1200–1500, Ca: 1000–1300, K: 4700–5100, Mg: 255–350, Fe: 5.0–23, Mn: 1.8–2.6 and Zn: 6.8–10.9 mg/day[43]), one gram of the dune wormwood's aqueous extracts could supply up 5% of Mn and 21% of Fe (with regard to the minimum reference values), without reaching the maximum recommended daily intake of Na, and therefore may contribute to the adult daily intakes of some major and minor elements. Moreover, values of Cu and Cr can be considered low and safe for consumption as they are below the recommended dietary allowance values (Cu: 700–1000, Cr: 20–45 µg/day)[43] and potentially toxic minerals like Ni, Cd and Pb were not detected (below the LOQs). Even if these were present in the extracts at undetected levels, they would not constitute a threat since the LOQs, when adjusted to the equivalent units based on the extraction yields (Table 1), are below legislated values for plants (Pb 0.3 µg/g and Cd 0.2 µg/g of plant material; EC Regulation 1881/2006). Overall, results highlight a possible nutritional role of the dune wormwood's extracts, particularly aerial-organs and aqueous extracts, as an additional mineral source.

Toxicological evaluation. The potential toxicity of new herbal products for human use, such as plant extracts, must be determined to establish its safe consumption. Preliminary toxicological evaluations can be made by *in vitro* models that address the sensitivity of mammalian cell lines to possible toxic effects of the extracts, delivering reliable and quick results and reducing *in vivo* testing[5–7,44,45]. Aiming at such a predictive toxicity screening, the dune wormwood extracts were tested for cytotoxicity towards three mammalian cell lines and the resulting cellular viabilities are presented in Fig. 2. The aqueous extracts showed overall low toxicity with cell viability values higher than those obtained for tinctures. Infusions and decoctions exerted no toxic effects in the hepatocarcinoma (HepG2) cells while tinctures had moderate to low toxicity with cellular viabilities between 62% (aerial-organs) and 72% (roots). For the microglia (N9) cell line, toxicity of the aerial-organs aqueous extracts was very low (>90% viabilities) while that of aerial-organs tincture (73% viability) and roots infusion and decoction (63–68% viabilities) can be considered moderate to low; roots' tincture exerted a more toxic effect with 50% of cellular viability. For the stromal (S17) cells, roots' aqueous extracts had low toxicity (71–73% viabilities) whereas roots' tincture (61% viability) and aerial-organs water extracts (66–68% viabilities) were only moderately toxic; aerial-organs' tincture resulted in 55% of cellular viability. As a preliminary safety evaluation of *A. campestris* subsp. *maritima* extracts, results suggest that they may be regarded as safe for consumption, although some caution is advised regarding the use of hydro-alcoholic extracts. Nevertheless, for comparison purposes, the widely consumed green tea had cellular viabilities as low as 30% in S17 cells[7]. Moreover, acute toxicity tests of *A. campestris* leaves aqueous extracts on mice showed that up to 3200 mg/kg body weight administered orally neither killed nor impaired behaviour[42] and intraperitoneal injections rendered a LD_{50} equivalent to 2500 mg/kg b.w.[28].

Biological activities. Antioxidants can be considered a group of medicinally preventive molecules also used as food additives to inhibit food oxidation. Hence, natural antioxidant sources are increasingly sought after as an alternative to synthetic antioxidants in the food, cosmetic and therapeutic industries[3,22]. Antioxidants are scavengers of free radicals or ROS and deactivators of metal catalysts by chelation, among other activities, reducing oxidative stress and consequent cell damage. It is increasingly documented that dietary antioxidant phytochemicals effectively prevent oxidative damage, reducing the risk of oxidative-stress related conditions like neurodegenerative and vascular diseases, carcinogenesis or inflammation[10,22,46]. Their intake is also associated with the management of diabetes mellitus[22] and amelioration of skin ageing conditions[47].

In this work, the antioxidant potential of the dune wormwood's extracts was assessed by eight different methods targeting radical scavenging activity (RSA) and metal-related potential (Table 5). The extracts were overall effective as scavengers of DPPH, ABTS, NO and $O_2^{\bullet-}$ radicals and at reducing iron, but their chelating properties were moderate for copper and low for iron. In the DPPH assay the aerial-organs' tincture had the lowest IC_{50} value (240 µg/mL), lower than that obtained for the positive control (BHT; IC_{50} = 320 µg/mL), followed by aerial-organs' infusion (330 µg/mL), decoction (340 µg/mL) and roots decoction (370 µg/mL), all similar to BHT

Samples	Organ	Extract	Antioxidant activity						
			DPPH	ABTS	NO	$O_2^{\bullet-}$	FRAP	CCA	ICA
A. campestris subsp. *maritima*	Roots	Infusion	0.39 ± 0.02^{c}	0.45 ± 0.02^{de}	0.74 ± 0.03^{c}	0.21 ± 0.01^{b}	0.29 ± 0.01^{c}	1.64 ± 0.10^{c}	7.82 ± 0.37^{d}
		Decoction	0.37 ± 0.02^{bc}	0.37 ± 0.01^{b}	0.55 ± 0.01^{bc}	0.18 ± 0.01^{a}	0.25 ± 0.00^{b}	1.64 ± 0.04^{c}	7.37 ± 0.34^{cd}
		Tincture	0.46 ± 0.02^{d}	0.46 ± 0.01^{e}	1.40 ± 0.07^{d}	0.33 ± 0.01^{d}	0.24 ± 0.00^{b}	3.60 ± 0.11^{e}	>10
	Aerial-organs	Infusion	0.33 ± 0.03^{b}	0.41 ± 0.01^{cd}	0.70 ± 0.03^{bc}	0.23 ± 0.01^{bc}	0.17 ± 0.00^{a}	1.31 ± 0.05^{b}	6.47 ± 0.35^{bc}
		Decoction	0.34 ± 0.03^{bc}	0.44 ± 0.01^{de}	0.49 ± 0.01^{ab}	0.24 ± 0.00^{c}	0.27 ± 0.01^{c}	1.30 ± 0.09^{b}	6.33 ± 0.43^{b}
		Tincture	0.24 ± 0.01^{a}	0.40 ± 0.01^{bc}	0.29 ± 0.02^{a}	0.35 ± 0.01^{e}	0.23 ± 0.01^{b}	2.51 ± 0.09^{d}	>10
BHT*			0.32 ± 0.02^{b}	0.11 ± 0.00^{a}			—		
Ascorbic acid*					2.31 ± 0.22^{e}				
Catechin*						0.62 ± 0.01^{f}			
EDTA*								0.13 ± 0.00^{a}	0.07 ± 0.00^{a}

Table 5. Antioxidant activity (IC_{50} values, mg/mL) of infusions, decoctions and tinctures from *Artemisia campestris* subsp. *maritima* organs: radical scavenging on DPPH, ABTS, NO and $O_2^{\bullet-}$ radicals, ferric reducing antioxidant power (FRAP) and metal-chelating activities on copper (CCA) and iron (ICA). Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each column different letters mean significant differences ($p < 0.05$). *Positive controls.

($p < 0.05$). High RSA against DPPH was also reported by Megdiche-Ksouri *et al.*[20] in methanolic extracts from shoots of the same *A. campestris* subspecies. Aerial-organs' tincture also had the strongest NO scavenging activity allowing an IC_{50} of 290 µg/mL, comparable to that of this organs' decoction (490 µg/mL, $p < 0.05$); most interestingly all extracts were better NO scavengers than the positive control (ascorbic acid, $IC_{50} = 2.31$ mg/mL). This was also the case with $O_2^{\bullet-}$ scavenging as catechin had the highest IC_{50} (620 µg/mL). For this radical's assay, however, the lowest IC_{50} value was obtained after the application of roots' decoction (180 µg/mL), followed by infusions from both organs (roots: 210 µg/mL, aerial-organs: 230 µg/mL). Roots decoction was also the best ABTS scavenger ($IC_{50} = 370$ µg/mL), statistically similar to the result obtained with the aerial-organs' tincture ($IC_{50} = 400$ µg/mL; $p < 0.05$). As for the iron reducing capacity, the best result was obtained with the aerial-organs' infusion with an IC_{50} of 170 µg/mL, followed by aerial-organs' tincture (230 µg/mL), roots tincture (240 µg/mL) and decoction (250 µg/mL). This is in accordance with Megdiche-Ksouri *et al.*[20] findings of a high FRAP in this subspecies. Conversely, the extracts iron-chelating activity was comparatively low, with IC_{50} values higher that 5 mg/mL, while the capacity to chelate copper was moderate (best $IC_{50} = 1.3$ mg/mL in aerial-organs' water extracts). Tannins were not detected in any of the extracts, which may partially explain its low chelating potential since tannins are known metal chelating agents[48]. The aerial-organ's water extracts had the highest capacity to chelate both metals (CCA, $IC_{50} = 1.30$–1.31 mg/mL; ICA, $IC_{50} = 6.33$–6.47 mg/mL). Several studies previously highlighted the high antioxidant capacity of similar aqueous and hydro-alcoholic extracts from *A. campestris*[27,29,30,32,33,42], which confirms our results of strong *in vitro* antioxidant potential for this subspecies. Most of these authors also credited the pronounced antioxidant activity of the extracts to the polyphenolic content which is, in fact, an association widely reported by several studies that confirm the phenolics' role as antioxidants, especially in halophyte plants[3]. Accordingly, aerial-organs' tincture had the highest levels of almost all phenolics groups (Table 1) and was also of the best-scoring extracts in terms of antioxidant activity. Actually, that extract also had overall higher abundance and variety of individual phenolic constituents (Tables 2 and 3), altogether corroborating the hypothesis that phenolics play a major role in the sample's strong antioxidant potential. For example, the main components quinic, chlorogenic and caffeic acids, determined in higher amounts in aerial-organs' tincture (Table 2), are known antioxidant compounds[49–51]. Nevertheless, roots' extracts showed greater relative abundances of some major constituents (Table 3), such as the dicaffeoylquinic acid (peak 25, Fig. 1) in roots' decoction, and quinic, chlorogenic and caffeic acids, although in lower levels than in aerial-organs' samples, were the predominant constituents. Synergistic and/or additive effects between these phytoconstituents may also account for the equally high antioxidant activity of roots' decoction.

Besides antioxidant activity, other bioactivities have been ascribed to extracts from *A. campestris* as for example hypoglycaemic effects[28]. Type 2 diabetes mellitus (T2DM) is a common health disorder characterized by high blood glucose levels that can lead to major metabolic complications if left untreated[52]. One effective strategy to manage T2DM is to inhibit carbohydrate-hydrolysing enzymes, such as α-glucosidase, delaying carbohydrate digestion and uptake and resulting in reduced postprandial blood glucose levels, therefore lowering hyperglycaemia linked to T2DM[52,53]. In this sense, the dune wormwood's extracts were tested for their capacity to inhibit microbial and mammalian α-glucosidases as an assessment of their anti-diabetic potential.

All extracts had the ability to inhibit the microbial α-glucosidase but the most active samples were roots' aqueous extracts and aerial-organs' decoction ($IC_{50} = 0.89$–1.13 mg/mL). Interestingly, all of the extracts were more efficient at inhibiting the microbial α-glucosidase than the positive control used acarbose ($IC_{50} = 3.14$ mg/mL), a clinically used inhibitor of this enzyme. However, only the roots' extracts were able to inhibit mammalian α-glucosidase, particularly roots' tincture ($IC_{50} = 2.90$ mg/mL), still more active than acarbose ($IC_{50} = 4.64$ mg/mL). Roots' extracts were less active towards the mammalian enzyme than for the microbial counterpart, an outcome already described for some compounds showing that enzyme origin can influence the extracts' inhibition of α-glucosidase[54]. Nevertheless, and despite the notion that the mammalian enzyme is a more reliable proxy for *in vivo* activity[54], the *in vivo* anti-diabetic potential of *A. campestris* aqueous extracts from leaves was demonstrated

Samples	Organ	Extract	Microbial α-glucosidase	Mammalian α-glucosidase	Tyrosinase
A. campestris subsp. *maritima*	Roots	Infusion	0.92 ± 0.04^{a}	6.09 ± 0.41^{c}	7.58 ± 0.14^{d}
		Decoction	0.89 ± 0.03^{a}	6.62 ± 0.48^{c}	5.56 ± 0.45^{c}
		Tincture	2.54 ± 0.05^{c}	2.90 ± 0.22^{a}	5.23 ± 0.12^{c}
	Aerial-organs	Infusion	1.64 ± 0.05^{b}	>10	4.13 ± 0.27^{b}
		Decoction	1.13 ± 0.03^{a}	>10	5.14 ± 0.35^{c}
		Tincture	1.62 ± 0.06^{b}	>10	5.35 ± 0.25^{c}
Acarbose*			3.14 ± 0.23^{d}	4.64 ± 0.76^{b}	
Arbutin*					0.48 ± 0.01^{a}

Table 6. Inhibitory activities (IC_{50} values, mg/mL) on microbial and mammalian α-glucosidase enzymes, and on tyrosinase enzyme of infusions, decoctions and tinctures from *A. campestris* subsp. *maritima* organs. Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each column different letters mean significant differences ($p < 0.05$). *Positive controls.

by Sefi *et al.*[28], having significantly reduced blood glucose levels in diabetic rats. Those authors considered that the *in vivo* hypoglycaemic activity of *A. campestris* extracts could be related to its strong antioxidant properties, and stated the role that this plant's water extracts can have on the treatment of diabetic patients[28]. It is recognized that polyphenolic compounds, besides potent antioxidants[3,10], can also have glucosidase-modulating activities therefore contributing to the management of T2DM[52]. The dune wormwood's extracts had a high phenolic content and contained some compounds with described hypoglycaemic activity, namely chlorogenic, caffeic and ferulic acids[50,51], and with reported α-glucosidase inhibitory activity, like isoquercitrin, luteolin, quercetin and apigenin[52]. Overall, our results suggest that all dune wormwood's extracts could be beneficial in managing T2DM by its capacity to inhibit dietary carbohydrate digestive enzymes, which was higher than acarbose, and consequently controlling glucose levels. Furthermore, as oxidative stress has been considered a mediator in diabetic complications[55], the extracts' strong antioxidant potential can also be an adjuvant in preventing or attenuating the disease's symptoms when used in combined anti-diabetic strategies.

Skin hyperpigmentation (e.g. melasma, freckles, age spots) is a result of melanin over-production but, as tyrosinase is essential in melanin biosynthesis, inhibition of this enzyme can help prevent and/or manage undesired skin darkening[47,56]. Tyrosinase is also responsible for unwanted browning of fruits and vegetables, which decreases their market value[56,57]. Hence, tyrosinase inhibitors from natural sources are increasingly sought not only for cosmetic and medicinal purposes but also for their potential in improving food quality[47,56,57]. In this context, the tyrosinase inhibitory potential of the dune wormwood's extracts was evaluated and results are depicted on Table 6. All extracts were active, particularly aerial-organs' infusion ($IC_{50} = 4.13$ mg/mL), although less effective than the used positive control (arbutin, $IC_{50} = 0.48$ mg/mL). Tyrosinase is a copper-containing enzyme[56] and thus the extracts' moderate copper chelating activity could be related to their tyrosinase inhibitory capacity. In fact, metal chelating and ROS-scavenging properties are mechanisms often thought to be related with the reducing activity of flavonoids[47]. Some flavonoids were already identified as tyrosinase inhibitors, as for example quercetin, kaempferol and taxifolin, the last being as effective as arbutin[57]. All these compounds were detected in the dune wormwood's extracts, possibly contributing to their tyrosinase inhibitory activity. To the best of our knowledge, this is the first report on the tyrosinase inhibitory potential of *A. campestris* subsp. *maritima*.

This study reports for the first time a comprehensive assessment of the biotechnological potential of *A. campestris* subsp. *maritima* as a source of innovative products with health promoting properties. Overall, our results point to the potential role of infusions, decoctions and tinctures of the dune wormwood in the prevention of oxidative-stress related diseases and in the management of diabetes and skin-hyperpigmentation conditions. More specifically, those formulations can be considered an unexplored source of polyphenolic and mineral constituents, antioxidants and α-glucosidase and tyrosinase inhibitors that could deliver raw material to different commercial segments including the pharmaceutical, cosmetic and/or food industries. Further studies are being pursued aiming to fully explore the health-promoting benefits of this plant's extracts, namely their *in vivo* effects.

Methods

Plant collection. *Artemisia campestris* L. subsp. *maritima* Arcang. (Compositae) plants were collected in South Portugal, within the area of the Ria Formosa coastal lagoon, near Faro (Ludo, 37°2′6.526″N 7°58′58.465″W), in June of 2013. The taxonomical classification was carried out by Dr. Manuel J. Pinto, botanist in the National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal, and a voucher specimen (voucher code MBH34) is kept in the herbarium of Marbiotech's laboratory. Plants were divided in roots and aerial-organs (stems and leaves), oven dried at 50 °C until complete dryness (3 days), milled and stored at −20 °C until use.

Extracts preparation: infusions, decoctions and tinctures. Water extracts were prepared similarly to a regular cup-of-tea: 1 g of dried plant material was homogenized in 200 mL of ultrapure water. For infusions, the biomass was immersed in boiling water for 5 min; for decoctions, the biomass was boiled in water for 5 min. Hydro-ethanolic extracts were prepared similarly to a home-made tincture: 20 g of dried plant material was left homogenising in 200 mL of 80% aqueous ethanol for a week. Independent extractions ($n \geq 3$) for each combination of method + plant-part were made. All extracts were filtered (Whatman n° 4), vacuum and/or freeze-dried

and stored in a dark, cool and moist-free environment. Extracts were re-suspended in water or aqueous ethanol to a concentration of 10 mg/mL to determine (spectrophotometric) phenolic content and test for bioactivities. For these assays, no significant differences were found among corresponding extracts from the different extractions and therefore freeze-dried extracts were pooled accordingly for the remaining analyses.

Phytochemical composition of the extracts. *Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content.* The TPC, TFC and CTC were estimated by spectrophotometric methods, respectively: Folin-Ciocalteau, aluminium chloride colorimetric and 4-dimethylaminocinnamaldehyde (DMACA), as described in Rodrigues *et al.*[26]. Gallic acid, quercetin and catechin were used as standards and results are presented as milligrams of standard equivalents per gram of extract dry weight (GAE, QE and CE, respectively; mg/g dw). Further information pertained to these methods is presented in Table S1 (supplementary material).

Hydroxycinnamic acid derivatives (HAD), flavonols and anthocyanins content. Total contents in HAD, flavonols and anthocyanins were assessed spectrophotometrically as described previously[26] using caffeic acid, quercetin and cyanidin chloride as standards, respectively. Results are presented as milligrams of standard equivalents per gram of extract dry weight (CAE, QE and CCE, respectively; mg/g dw). Further information pertained to these methods is presented in Table S1 (sup. material).

Profile of moderately polar compounds by UHPLC. Standard stock solutions were prepared at 1 mg/mL in UHPLC-grade methanol and stored at 4 °C in the dark. Standard dilutions were prepared in 60:40 (*v:v*) methanol:40 mM ammonium formate buffer (reference standards: apigenin, apigenin-7-O-glucoside (apigetrin), catechin, cyanidin-3-O-arabinoside, cyanidin-3-O-galactoside chloride (ideain chloride), cyanidin-3-O-glucoside chloride (kuromanin chloride), cyanidin-3-O-rutinoside chloride (keracyanin chloride), (+)-dihydrokaempferol ((+)-aromadendrin), epicatechin, epigallocatechin, epigallocatechin gallate, flavone, galangin, hesperidin, hesperidin methyl chalcone, 4-hydroxybenzaldehyde, kaempferol, kaempferol-3-O-glucoside (astragalin), limonin, luteolin, naringenin, naringin, neohesperidin dihydrochalcone, phloretin, phloretin-O-20-glucoside (phloridzin), procyanidin B2, protocatechuic acid, propyl gallate, quercetin, quercetin-3-O-arabinoside (avicularin), quercetin-3-O-galactoside (hyperin), quercetin-3-O-glucoside (isoquercitrin), quercetin-3-O-rhamnoside (quercitrin), rutin, uvaol, and caffeic, chlorogenic, coumaric, dihydrocaffeic, ellagic, ferulic, gallic, gentisic, m-hydroxybenzoic, hydroferulic, p-hydroxybenzoic, oleanolic, quinic, rosmarinic, salicylic, sinapinic and syringic acids). Freeze-dried pooled extracts (approx. 15 mg) were dissolved in 20 mL of 60:40 methanol:water +40 mM ammonium formate, followed by 1 min vortex mixing, 30 min sonication (40 kHz, 100 W, room temperature) and 10 min centrifugation (3000 rpm). Supernatants were diluted 100-fold and stored along with undiluted extracts at 4 °C, until analysis. Both undiluted and diluted extracts were analysed with a generic ultra-high performance liquid chromatography – photodiode array – accurate mass mass spectrometry (UHPLC-PDA-amMS) method for moderately polar phytochemicals adapted from De Paepe *et al.*[34] and fully detailed in Pereira *et al.*[5]. Briefly, for analysis 5 µL of extract was injected on an UPLC BEH SHIELD RP18 column (3.0 mm × 150 mm, 1.7 µm; Waters, MA) and thermostatically eluted (40 °C) with a quaternary solvent manager and a 'Hot Pocket' column oven. The mobile phase consisted of water +0.1% formic acid (A) and acetonitrile +0.1% formic acid (B), following a gradient of (min/%A): 0.0/100, 9.91/74, 18.51/35, 18.76/0, 23.76/0, 23.88/100, 26.00/100. For detection, a Q Exactive MS (Thermo Fisher Scientific, Bremen, Germany) was used with heated electrospray ionization (HESI). For quantitative analysis, full scan data were acquired using polarity switching with a mass/charge (*m/z*) range of 120–1800 and resolving power set at 70 000 at full width at half maximum (FWHM). Data were also recorded using data dependent fragmentation ($ddMS^2$) in positive and negative ionization mode to obtain additional structural information. The PDA detector was set to scan from 190 to 800 nm during all analyses. The lowest calibration point included in the calibration curve was used to calculate the limits of quantitation (LOQs). The concentration ranges described by De Paepe *et al.*[34] were also used during the present work. Results regarding concentrations of identified compounds were calculated as µg/mg of extract dry weight.

Mineral composition. Freeze-dried pooled extracts were digested in a combination of nitric acid (HNO_3) and hydrogen peroxide on a hot plate and evaporated until dryness (up to 24 h). Digested samples were diluted in 20 mL of 5% HNO_3 and analysed for mineral content by Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), as described in Pereira *et al.*[6]. Instrumental detection limits were as follows: Ca: 0.04 µg/L, Cd: 1.4 µg/L, Cr: 0.3 µg/L, Cu: 0.5 µg/L, Fe: 1.7 µg/L, K: 0.6 µg/L, Mg: 0.031 mg/L, Mn: 0.1 µg/L, Na: 0.1 µg/L, Ni: 1.1 µg/L, Pb: 2.5 µg/L and Zn: 3.1 µg/L. Results were expressed as mg or µg/g of extract dry weight (DW). Appropriate blanks were also produced and analysed.

Toxicological evaluation of the samples. Samples' toxicity was assessed using murine microglia (N9), murine bone marrow stromal (S17) and human hepatocellular carcinoma (HepG2) cell lines. The N9 cell line was provided by the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal), S17 and HepG2 cells were delivered by the Centre for Biomedical Research (CBMR, University of Algarve, Portugal). Cell culture was maintained as described in Pereira *et al.*[6]. Toxicity was evaluated according to Rodrigues *et al.*[7]. Briefly, N9 cells where plated at an initial density of 1×0^4 cells/well while S17 and HepG2 cells were seeded at 5×10^3 cells/well, all in 96-well plates. Freeze-dried pooled extracts were dissolved in culture medium (100 µg/mL) and incubated with cells for 72 h; culture medium was used as negative control and hydrogen peroxide (H_2O_2) as positive control. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and results were expressed in terms of cell viability (%).

Biological activities. *Antioxidant activity assessed by four radical-based assays.* The extracts' radical scavenging capacity against the DPPH (1,1-diphenyl-2picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), NO (nitric oxide) and $O_2^{\bullet-}$ (superoxide) radicals was assessed as described in Rodrigues *et al.*[7,26]. BHT (butylated hydroxytoluene), ascorbic acid and catechin were used as positive controls. Results were calculated as percentage of antioxidant activity in relation to a control containing ultrapure water or aqueous ethanol, and expressed as IC_{50} values (mg/mL; half maximal inhibitory concentration, ascertained for extracts with activities higher than 50% at 10 mg/mL).

Antioxidant activity assessed by three metal-related assays. The extracts' chelating ability towards copper (CCA) and iron (ICA) and their Fe^{3+} reducing capacity (ferric reducing antioxidant power, FRAP) were assessed as described previously[26]. EDTA (ethylenediamine tetraacetic acid) and BHT were used as positive controls. Results were calculated as percentage of antioxidant activity relative to a positive control for FRAP, and in relation to a negative control (ultrapure water/aqueous ethanol) for CCA and ICA, and were expressed as IC_{50} values (mg/mL).

In vitro anti-diabetic activity: inhibition of microbial and mammalian α-glucosidases. The microbial α-glucosidase enzyme was obtained from the yeast *Saccharomyces cerevisiae*; rat's intestine acetone powder was used to obtain a crude enzyme extract as an example of a mammalian-origin α-glucosidase. The extracts' capacity to inhibit both enzymes was assessed following Kwon *et al.*[53] and using acarbose as positive control. Results are expressed as IC_{50} values (mg/mL), calculated as percentage of inhibitory activity in relation to a control (ultrapure water/aqueous ethanol).

In vitro tyrosinase inhibition. The extracts' ability to inhibit tyrosinase was assessed following Custódio *et al.*[58], using arbutin as positive control. Results, calculated as percentage of inhibitory activity in relation to a control (ultrapure water/aqueous ethanol), are expressed as IC_{50} values (mg/mL).

Statistical analysis. Experiments were conducted at least in triplicate and results were expressed as mean ± standard deviation (SD). Significant differences ($p < 0.05$) were assessed by one-way analysis of variance (ANOVA) followed by Tukey pairwise multiple comparison test or, when parametricity of data did not prevail, Kruskal Wallis one-way analysis of variance on ranks followed by Dunn's test. Statistical analyses were executed using XLStat® version 19.4. IC_{50} values were computed by curve fitting in GraphPad Prism® version 6.0c.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

C.G.P., L.C. and L.B. designed the study. S.B. analysed the phytochemical profile of the extracts by LC-PDA-amMS; C.M. performed the toxicological evaluation of the samples; T.F.S. assessed the extracts' mineral contents; C.G.P. undertook the remaining work. C.G.P. wrote the main manuscript text with the contribution of L.C., S.B., L.P., J.V. and L.B. L.C. and L.B. jointly supervised the work. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

Table S1. Information pertained to the quantification of total polyphenol content (TPC), total flavonoid content (TFC) condensed tannin content (CTC), hydroxycinnamic acid derivatives (HAD), flavonols and anthocyanins: calibration curve equations, linearity, detection limits and limits of quantification.

	CCE	Linearity	DL (mg/mL)	LOQ (mg/g DW)
TPC[1]	y = 1.2800x + 0.1287	R^2 = 0.9882	0.006	2.067 [1]
TFC[2]	y = 3.4394x + 0.1132	R^2 = 0.9940	0.002	0.599 [2]
CTC[3]	y = 1.0453x + 0.1385	R^2 = 0.9857	0.002	0.781 [3]
HAD[4]	y = 3.9839x + 0.3250	R^2 = 0.9984	0.005	1.534 [4]
Flavonols[5]	y = 3.3706x + 0.1116	R^2 = 0.9990	0.003	1.146 [5]
Anthocyanins[6]	y = 15813x + 0.0769	R^2 = 0.9808	0.001	0.316 [6]

CCE: Calibration curve equation; DL: Detection limits; LOQ: Limit of quantification
[1] mg GAE/g DW, GAE: gallic acid equivalents
[2] mg QE/g DW, QE: quercetin equivalents
[3] mg CE/g DW, CE: catechin equivalents
[4] mg CAE/g DW, CAE: caffeic acid equivalent
[5] mg QE/g DW, QE: quercetin equivalents
[6] mg CCE/g DW, CCE: cyanidin chloride equivalents

Table S2. Chromatographic and spectral data of the tentatively identified compounds in infusions, decoctions and tinctures from *A. campestris* subsp. *maritima* organs, detected with a generic LC-PDA-amMS method for moderately polar phytochemicals.

Compound tentative ID	Molecular formula	HESI neg full MS	HESI neg ddMS^2	HESI pos full MS	HESI pos ddMS^2	*RT (min)	Max UV absorb. (nm)	Previously reported
Chlorogenic acid isomer (isochlorogenic acid A, B or C)	$C_{16}H_{18}O_9$	353.08804 $[M-H]^-$	191.1; 179.0; 173.0; 135.0	355.10217 $[M+H]^+$; 377.08395 $[M+Na]^+$	163.0; 135.0	7.77	300; 326	[1][2]
Hydroxybenzoic acid isomer (2,3-Dihydroxybenzaldehyde)	$C_7H_6O_3$	137.02452 $[M-H]^-$	119.0; 109.0; 108.0; 93.0; 81.0	139.03917 $[M+H]^+$	111.0; 93.0; 65.0	8	-	-
Hexoside of scopoletin (scopolin)	$C_{16}H_{18}O_9$	399.09436 $[M-H+FA]^-$	191.0; 176.0; 148.02	372.12912 $[M+NH_4]^+$; 377.08454 $[M+Na]^+$	193.0; 178.0; 165.0; 133.0	8.48	284; 339	[2][3]
Hexoside of coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin)	$C_{17}H_{20}O_{10}$	429.10516 $[M-H+FA]^-$; 383.09921 $[M-H]^-$	221.0; 206.0; 191.0; 163.0	402.13951 $[M+NH_4]^+$; 407.09479 $[M+Na]^+$; 791.20083 $[2M+Na]^+$; 786.24508 $[2M+NH_4]^+$	223.1; 208.0; 190.0; 135.0	9.02	291; 327	-
Chlorogenic acid isomer (isochlorogenic acid A, B or C)	$C_{16}H_{18}O_9$	353.08804 $[M-H]^-$	191.1; 179.0; 173.0; 135.0	355.10217 $[M+H]^+$; 377.08395 $[M+Na]^+$	163.0; 135.0	9.33	300; 326	[1][2]
Aesculetin	$C_9H_6O_4$	177.01996 $[M-H]^-$	149.0; 133.0; 105.0	179.03399 $[M+H]^+$	151.0; 133.0; 123.0	9.61	-	[4]
Chlorogenic acid isomer (isochlorogenic acid A, B or C)	$C_{16}H_{18}O_9$	353.08804 $[M-H]^-$	191.1; 179.0; 173.0; 135.0	355.10217 $[M+H]^+$; 377.08395 $[M+Na]^+$	163.0; 135.0	9.88	-	[1][2]
Fraxetin	$C_{10}H_8O_5$	207.03047 $[M-H]^-$	192	209.04435 $[M+H]^+$; 231.02634 $[M+Na]^+$	209.0; 194.0; 181.0; 163.0; 153.0; 149.0; 135.0	10.19	-	-
Coumaric acid hexoside isomer	$C_{15}H_{18}O_8$	325.09327 $[M-H]^-$; 371.09916 $[M-H+FA]^-$	163.0; 119.0	327.10744 $[M+H]^+$; 344.13401 $[M+NH_4]^+$; 349.08935 $[M+Na]^+$	165.1; 147.0	10.32	276; 313	-
Coumaric acid hexoside isomer	$C_{15}H_{18}O_8$	325.09327 $[M-H]^-$; 371.09916 $[M-H+FA]^-$	163.0; 119.0	327.10744 $[M+H]^+$; 344.13401 $[M+NH_4]^+$; 349.08935 $[M+Na]^+$	-	10.76	-	-
Coumarin sulfate with 2 methoxy moieties (iso-fraxidin or fraxidin)	$C_{11}H_{10}O_8S$	301.00267 $[M-H]^-$	221.0; 206.0; 191.0; 163.0	303.01702 $[M+H]^+$	223.1; 208.0; 190.0; 135.0	11.54	292; 334	-
Coumarin sulfate (fraxetin-*O*-sulfate isomer)	$C_{10}H_8O_8S$	286.98758 $[M-H]^-$	207.0; 192.0	289.00137 $[M+H]^+$	209.0; 194.0; 181.0; 163.0; 153.0; 149.0; 135.0	11.65	296; 342	-
Not identified	$C_{12}H_{18}O_7S$	305.07022 $[M-H]^-$	225.1; 181.1; 147.1; 97.0	-	-	11.74	-	-
Coumarin sulfate (fraxetin-*O*-sulfate isomer)	$C_{10}H_8O_8S$	286.98758 $[M-H]^-$	207.0; 192.0	289.00137 $[M+H]^+$	209.0; 194.0; 181.0; 163.0; 153.0; 149.0; 135.0	11.78	300; 341	-
Scopoletin	$C_{10}H_8O_4$	191.03563 $[M-H]^-$	176.0; 148.0; 104.0	193.04921 $[M+H]^+$	178.0; 133.0	12.03	301; 336	[4]
Coumarin with 2 methoxy moieties (iso-fraxidin or fraxidin)	$C_{11}H_{10}O_5$	221.04623 $[M-H]^-$	206.0; 191.0; 163.0	223.06034 $[M+H]^+$; 245.04239 $[M+Na]^+$	208.0; 190.0; 135.0	12.11	300; 341	[4]
Coumarin sulfate (scopoletin-*O*-sulfate isomer)	$C_{10}H_8O_7S$	270.99296 $[M-H]^-$	191.0; 176.0; 148.0	273.00636 $[M+H]^+$	193.0; 178.0; 165.0; 149.1; 133.0	12.19	282; 339	-
Fraxidin-caffeoyl-hexoside	$C_{26}H_{26}O_{13}$	545.13217 $[M-H]^-$	323.1; 221.0; 206.0; 191.0; 179.0; 163.0; 135.0	547.14421 $[M+H]^+$; 564.17128 $[M+NH_4]^+$; 569.12661 $[M+Na]^+$	325.1; 223.1; 208.0; 190.0; 181.0; 135.0	13.03	297; 328	-
Methoxy-cinnamic acid	$C_{10}H_{10}O_3$	177.05635 $[M-H]^-$	162.0; 134.0	179.07054 $[M+H]^+$	161.1; 147.0; 133.1; 119.0; 105.1; 91.0	13.53	-	[2][5]
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	13.65	300; 325	[4]

Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	14.06	300; 328	[4]
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	-	531.14914 $[M+H]^+$	-	14.35	-	-
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	14.45	300; 328	[4]
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 353.1; 191.1; 179.0; 173.0; 161.0; 135.0	531.14914 $[M+H]^+$	177.0; 163.0; 145.0; 135.0; 117.0	14.6	-	-
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	14.79	300; 325	[4]
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 353.1; 191.1; 179.0; 173.0; 161.0; 135.0	531.14914 $[M+H]^+$	177.0; 163.0; 145.0; 135.0; 117.0	15.03	-	-
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 353.1; 191.1; 179.0; 173.0; 161.0; 135.0	531.14914 $[M+H]^+$	177.0; 163.0; 145.0; 135.0; 117.0	15.37	-	-
Caffeic acid coupled to $C_{11}H_{12}O_6$	$C_{20}H_{18}O_9$	401.08864 $[M-H]^-$	239.1; 179.0; 149.1; 135.0	403.1027 $[M+H]^+$; 420.12903 $[M+NH_4]^+$; 425.08439 $[M+Na]^+$	163.0; 135.0	15.89	304; 325	-
Flavonoid	$C_{15}H_{12}O_6$	287.05684 $[M-H]^-$	151.0; 135.0; 125.0; 107.0	289.07104 $[M+H]^+$	179.0; 163.0; 153.0; 135.0	15.99	286	-
Ethoxy or Dimethoxycinnamic acid	$C_{11}H_{12}O_4$	207.06657 $[M-H]^-$	179.0; 161.0; 135.0; 133.0	209.0811 $[M+H]^+$	181.0; 163.0; 145.0; 135.0; 117.0	16.1	295; 325	-
Tricaffeoylquinic acid	$C_{34}H_{30}O_{15}$	677.15139 $[M-H]^-$	515.1; 353.1; 335.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	679.16635 $[M+H]^+$; 696.19325 $[M+NH_4]^+$; 701.14839 $[M+Na]^+$	499.1; 163.0; 145.0; 135.0	16.13	295; 329	-
Dimethoxyflavonoid (axillarin)	$C_{17}H_{14}O_8$	345.0621 $[M-H]^-$	330.0; 315.0; 287.0; 259.0; 149.0	347.07648 $[M+H]^+$	332.0; 317.0; 289.0; 261.0; 186.0; 168.0	16.41	271; 349	-
Methoxyflavonoid (tamarixetin, rhamnetin, eupafolin, quercetin-3-methylether)	$C_{16}H_{12}O_7$	315.05118 $[M-H]^-$	300.0; 228.0	317.0661 $[M+H]^+$	302.0; 168.0	16.72	272; 345	[4]
Methoxyflavonoid (laricitrin or mearnsetin)	$C_{16}H_{12}O_8$	331.04646 $[M-H]^-$	316.0; 287.0; 271.0; 243.0; 209.0; 181.0; 166.1	333.06097 $[M+H]^+$	318.0; 290.0; 244.0	16.8	-	-
Trimethoxyflavonoid	$C_{18}H_{16}O_8$	359.07785 $[M-H]^-$	344.0; 329.0; 314.0; 301.0; 286.0; 258.0; 242.0; 214.0	361.09201 $[M+H]^+$	346.1; 330.0; 317.1; 300.1; 285.0; 275.0; 257.0; 229.0; 187.0; 169.0	17.49	-	-
Dimethoxyflavonoid (cirsiliol)	$C_{17}H_{14}O_7$	329.06699 $[M-H]^-$	314.0; 299.0; 271.0; 255.0; 227.0; 199.0	331.08188 $[M+H]^+$	316.1; 301.0; 273.0; 245.0; 186.0; 168.0	17.97	273; 345	-
Trimethoxyflavonoid	$C_{18}H_{16}O_8$	359.07785 $[M-H]^-$	344.0; 329.0; 314.0; 301.0; 286.0; 258.0; 242.0; 214.0	361.09201 $[M+H]^+$	346.1; 330.0; 317.1; 300.1; 285.0; 275.0; 257.0; 229.0; 187.0; 169.0	18.13	275; 329	-
Methoxyflavonoid (hispidulin)	$C_{16}H_{12}O_6$	299.05618 $[M-H]^-$	284	301.07092 $[M+H]^+$	286.0; 168.0	18.26	273; 329	[6]
Trimethoxyflavonoid (cirsilineol or eupatorin)	$C_{18}H_{16}O_7$	343.08286 $[M-H]^-$	328.1; 313.0; 298.0; 285.0; 270.0; 242.0; 163.0	345.09719 $[M+H]^+$	330.1; 315.0; 287.0; 169.0	18.89	272; 347	[4]
Tetramethoxyflavonoid	$C_{19}H_{18}O_8$	373.09376 $[M-H]^-$	358.1; 343.0; 328.0; 313.0; 300.0; 285.0; 257.0; 241.0; 177.1; 163.0	375.1073 $[M+H]^+$	360.1; 327.0; 314.1; 299.1; 285.0; 271.1; 257.0; 243.1; 229.0; 169.0	19.25	281; 327	-
Trimethoxyflavonoid (cirsilineol or eupatorin)	$C_{18}H_{16}O_7$	343.08286 $[M-H]^-$	328.1; 313.0; 299.1; 285.0; 270.0; 242.0; 163.0	345.09719 $[M+H]^+$	329.1; 315.0; 284.1; 255.1	19.48	-	[4]
Dimethoxyflavonoid (cirsimaritin)	$C_{17}H_{14}O_6$	313.07203 $[M-H]^-$	298.0; 283.0; 255.0; 227.0; 163.0	315.08657 $[M+H]^+$	300.1; 285.0; 168.0; 135.0	20.07	272; 335	[4]
Linderoflavone B	$C_{20}H_{18}O_8$	385.09385 $[M-H]^-$	-	387.10797 $[M+H]^+$	-	21.2	-	[4]

*RT – retention times

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CHAPTER 4

SEARCHING FOR NEW SOURCES OF INNOVATIVE PRODUCTS FOR THE FOOD INDUSTRY WITHIN HALOPHYTE AROMATIC PLANTS: *IN VITRO* ANTIOXIDANT ACTIVITY AND PHENOLIC AND MINERAL CONTENTS OF INFUSIONS AND DECOCTIONS OF *CRITHMUM MARITIMUM*

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Searching for new sources of innovative products for the food industry within halophyte aromatic plants: *In vitro* antioxidant activity and phenolic and mineral contents of infusions and decoctions of *Crithmum maritimum* L.

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ABSTRACT

Aromatic halophyte plants are an outstanding source of bioactive compounds and natural products with potential use in the food industry. This work reports the *in vitro* antioxidant activity, toxicity, polyphenolic profile and mineral contents of infusions and decoctions from stems, leaves and flowers of *Crithmum maritimum* L., an aromatic and edible maritime halophyte (sea fennel). *Aspalathus linearis* (Burm.f.) Dahlg. (rooibos) herbal tea was used as a reference. Sea fennel's tisanes, particularly from leaves, were rich in phenolic compounds and five of them (*p*-hydroxybenzoic and ferulic acids, epicatechin, pyrocatechol and 4-hydroxybenzaldehyde) were here described in *C. maritimum* for the first time. Chlorogenic acid was the dominant phenolic determined. Na was the most abundant mineral in all tisanes followed by Ca and Mg in leaves' tisanes and K in flowers. Sea fennel's samples had a similar antioxidant activity than those from *A. linearis*, and had no significant toxicity towards four different mammalian cell lines. Altogether, our results suggest that sea fennel can be a source of products and/or molecules for the food industry with antioxidant properties and minerals in the form, for example, of innovative health-promoting herbal beverages.

1. Introduction

Crithmum maritimum L., commonly known as sea fennel or rock samphire, is an aromatic, edible and medicinal halophyte common in marine coastal ecosystems along the European and North-African Atlantic, Mediterranean and Black Sea (Atia et al., 2011; Castroviejo et al., 2003). Sea fennel belongs to the same family (Apiaceae) as parsley and celery and has interesting sensory attributes: a slight salty taste with notes of celery, common fennel and peel of green citrus, followed by a strong aftertaste (Renna and Gonnella, 2012). In fact, it is traditionally used in countries such as Italy or Greece as an ingredient in salads, soups, sauces, as pickle

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ANOVA, one-way analysis of variance; BHT, butylated hydroxytoluene; CA, coumaric acid; CAE, caffeic acid equivalents; CCA, copper chelating activity; CE, catechin equivalents; CGA, chlorogenic acid; CTC, condensed tannin content; DMACA, 4-dimethylaminocinnamaldehyde; DPPH, 1,1-diphenyl-2picrylhydrazyl; DW, dry weight; FA, ferulic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; HAD, Hydroxycinnamic acid derivatives; HepG2, human hepatocellular carcinoma cells; HNO_3, nitric acid; ICA, iron chelating activity; LOQ, limit of quantitation; MP-AES, Microwave Plasma-Atomic Emission Spectrometer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N9, murine microglia cells; NCGA, neochlorogenic acid; NO, nitric oxide; QE, quercetin equivalents; RE, rutin equivalents; ROS, reactive oxygen species; RSA, radical scavenging activity; S17, murine bone marrow stromal cells; SD, standard deviation; SH-SY5Y, human neuroblastoma cells; TFC, total flavonoid content; TPC, total polyphenolic content.

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or spice and is acknowledged as a rich source of minerals and vitamin C (Castroviejo et al., 2003; Franke, 1982; Renna and Gonnella, 2012). Sea fennel has also folk therapeutic uses as an appetizer, tonic, purgative, carminative, anthelmintic or to prevent scurvy. Moreover, infusions and decoctions of the plant's aerial parts are used as diuretic, to treat renal and urinary complaints, digestive disorders, colic and inflammation of the urinary tract and prostate (Atia et al., 2011; Cornara et al., 2009; Franke, 1982).

Sea fennel is an important local resource in many coastal populations of the Mediterranean since ancient times; the first European farmers made it part of their diet and nowadays it is still consumed in many areas across Europe (Atia et al., 2011; Franke, 1982). Considering a growing world population coupled to global climate change, it is imperative to find alternative food resources that can overcome the threat of soil salinization for agriculture. In this sense, edible halophytes like sea fennel that can be cultivated in marine-influenced environments may be potential alternative crops. And, so far, few salt-tolerant plants have had their potential explored although halophyte products have recently draw some attention in food markets throughout the world (Atia et al., 2011; Buhmann and Papenbrock, 2013; Ventura and Sagi, 2013). For example, *Salicornia* species are currently trending in the gourmet food market and sea buckhorn (*Hippophae rhamnoides* L.) is sold in specialty stores as constituent in functional beverages or as herbal tea (Barreira et al., 2017; Gruenwald, 2009; Ventura and Sagi, 2013).

Herbal teas (tisanes made from plants other than *Camellia sinensis* L.) are worldwide popular beverages with a multitude of attributed health benefits (Patel, 2013; Pohl et al., 2016). Such is the case of rooibos tea (*Aspalathus linearis*) promoted for its high antioxidant potential (Joubert and de Beer, 2011). The health benefits of herbal beverages are mostly related to their high polyphenolic content and they are reported as a great source of these bioactive phytochemicals in our diet, as well as a potential mineral source (Gruenwald, 2009; Pohl et al., 2016). Phenolic compounds have recognized antioxidant properties and, given that oxidative stress is an underlying cause for several degenerative diseases, they can have beneficial outcomes in some health challenges like diabetes or neurodegenerative disorders (Lu and Yen, 2015; Sindhi et al., 2013). Medicinal and aromatic halophyte plants, such as sea fennel, combine a pleasant taste to potential health benefits and can be explored as sources of innovative bioactive compounds and/or products for the food industry as, for example, herbal beverages (Gruenwald, 2009). Such an approach on medicinal plants to unveil their functional properties and constituents, and explore their application as food products has been made with different plant species, as for example *Lycium barbarum* L., *Schisandra chinensis* (Turcz.) Baill and *Euphorbia denticulata* Lam. (Zengin et al., 2017; Mocan et al., 2016, 2017).

Research regarding sea fennel involves mainly organic extracts and *in vitro* studies report antioxidant and antimicrobial activities along with different groups of bioactive molecules, like phenolic acids and flavonoids (Atia et al., 2011; Buhmann and Papenbrock, 2013). Nonetheless, infusions made with flower tops and stalks of sea fennel collected in Croatia exhibited a high *in vitro* antioxidant activity and were rich in phenolic compounds (Siracusa et al., 2011). In this context this work reports, for the first time, a comparative evaluation of the *in vitro* antioxidant potential (using eight complementary assays) and the polyphenolic profile and mineral content of infusions and decoctions made with stems, leaves and flowers of sea fennel collected in the Alentejo coast of Portugal. We also report a preliminary *in vitro* toxicological evaluation using mammalian cells. The rooibos herbal tea was used as a comparison since it is one of the most consumed tea beverages worldwide.

2. Materials and methods

2.1. Plant collection

Crithmum maritimum L. plants were collected in Alentejo coast in Aljezur beach, (37°20′30.7″N 8°51′06.0″W) in August of 2013. The botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) performed the taxonomical classification. The Marbiotech laboratory keeps an herbarium with a voucher specimen (voucher code MBH33). Plants were divided in organs, namely stems, leaves and flowers, which were oven dried at 50 °C until complete dryness, milled and stored until use at −20 °C. Dried leaves of rooibos tea (*Aspalathus linearis* (Burm.f.) Dahlg., produced in Cape Town, South Africa) were bought in a regional supermarket, milled and stored at −20 °C.

2.2. Extracts preparation: "cup-of-tea" infusions and decoctions

Water extracts were prepared to equal a cup-of-tea: 1 g of dried plant material for 200 mL of ultrapure water. To prepare infusions, the biomass was immersed in boiling water for 5 min; for decoctions, the biomass was boiled in water for 5 min. Extracts were filtered (Whatman n° 4) and aliquots stored at −20 °C until use; some were freeze-dried for yield determination, high performance liquid chromatography (HPLC) and mineral analysis.

2.3. Phytochemical composition of the extracts

2.3.1. Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content

TPC was estimated by the Folin-Ciocalteau method, measuring the absorbance at 725 nm and using gallic acid as a standard. Results were presented as milligrams of gallic acid equivalents per cup-of-tea (mg GAE/200 mL). TFC was assessed by the aluminium chloride colorimetric assay with the absorbance measured at 510 nm and rutin used as standard. Results were calculated as rutin equivalents per cup-of-tea (mg RE/200 mL). CTC was determined by the assay 4-dimethylaminocinnamaldehyde (DMACA); the absorbance was measured at 640 nm using catechin as standard. Results were presented as catechin equivalents per cup-of-tea (mg CE/200 mL). All methods are described in Rodrigues et al. (2015).

2.4. Hydroxycinnamic acid derivatives (HAD) and flavonols content

HAD and flavonols were determined by the method reported in Rodrigues et al. (2015). Absorbances were measured at 320 nm and 360 nm, using caffeic acid and quercetin as standards, to estimate HAD and flavonols, respectively. Results were calculated as standard equivalents per cup-of-tea (CAE and QE, respectively; mg/200 mL).

2.5. Phenolic composition by high performance liquid chromatography – diode array detection (HPLC–DAD)

Freeze-dried extracts were dissolved at a concentration of 10 mg/mL in ultrapure water and analysed by HPLC–DAD according to the method and equipment already described by Rodrigues et al. (2015). Concentration of the several compounds were calculated using calibration curves prepared individually for each commercial standard dissolved in methanol (4-hydroxybenzaldehyde, apigenin, catechin hydrate, epicatechin, epigallocatechin, epigallocatechin gallate, pyrocatechol, quercetin, and caffeic, caffeoyquinic, chlorogenic, coumaric, ferulic, gallic, gentisic, *p*-hydroxybenzoic, neochlorogenic, rosmarinic, salicylic, syringic and

vanillic acids) and diluted to the required concentrations in ultrapure water. Results were calculated as mg per cup-of-tea (mg/200 mL) based on the extracts' yield.

2.6. Mineral composition

Freeze-dried extracts were analysed for mineral content by Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), after dry ashing the samples for 8 h, ash dissolution in hot nitric acid (HNO_3) and in hydrogen peroxide followed by sample dilution in 5% HNO_3. Working standards of different concentrations were prepared from certified standard solutions; for analytical quality assurance results were corrected by subtracting a blank from the analysed metal concentrations and samples were analysed in triplicate. Quantification wavelengths and calibration curves were selected to obtain the highest signal ratio and the lowest interference for the target elements. Spiking-and-recovery readings were carried out to assess validity of the results. Instrumental detection limits were: Ca, 0.04 μg/L; Cd, 1.4 μg/L; Cr, 0.3 μg/L; Cu, 0.5 μg/L; Fe, 1.7 μg/L; K, 0.6 μg/L; Mg, 0.031 mg/L; Mn, 0.1 μg/L; Na, 0.1 μg/L; Ni, 1.1 μg/L; Pb, 2.5 μg/L and Zn, 3.1 μg/L. Results were expressed as mg/cup-of-tea (mg/200 mL) based on the extracts' yield.

2.7. Toxicological evaluation of the samples

Murine microglia (N9) cell line was provided by the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal); murine bone marrow stromal (S17) and human hepatocellular carcinoma (HepG2) cell lines were obtained from the Centre for Biomedical Research (CBMR, University of Algarve, Portugal); human neuroblastoma (SH-SY5Y) cell line was obtained from Barcelona Science Park, Spain. RPMI-1640 culture medium was used to maintain N9 cells, while DMEM medium was used for HepG2, S17 and SH-SY5Y cells; both mediums were supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine (2 mM) and 1% penicillin (50 U/mL)/streptomycin (50 μg/mL). Cells were grown in an incubator in humidified atmosphere at 37 °C and 5% CO_2. Extracts' toxicity was assessed following Rodrigues et al. (2016). Briefly, S17 and HepG2 cells were plated at an initial density of 5×10^3 cells/well while N9 and SH-SY5Y cells where seeded at 1×0^4 cells/well, in 96-well plates. Freeze-dried extracts at 100 μg/mL were directly dissolved in culture medium and applied for 72 h; cells incubated with only culture medium were used as negative control and hydrogen peroxide (H_2O_2) was used as positive control for cell toxicity. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (absorbance at 590 nm) and results were expressed in terms of % cell viability.

2.8. Antioxidant activity

2.8.1. Determination of antioxidant activity by five radical-based assays

The scavenging capacity of the aqueous extracts against the radicals DPPH (1,1-diphenyl-2picrylhydrazyl), NO (nitric oxide), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), $O_2^{\cdot-}$ (superoxide) and $OH^{\cdot}$ (hydroxyl) was assessed according to Rodrigues et al. (2015, 2016) using butylated hydroxytoluene (BHT), catechin or ascorbic acid as positive controls. Results were calculated relative to a control containing ultrapure water, as percentage of antioxidant activity in a cup-of-tea.

2.8.2. Determination of antioxidant activity by three metal-related methods

The extracts' copper and iron chelating activities (CCA and ICA, respectively) and their ability to reduce Fe^{3+} (ferric reducing antioxidant power - FRAP) were evaluated as described previously (Rodrigues et al., 2015) using BHT and ethylenediamine tetraacetic acid (EDTA) as positive controls. Results were presented as percentage of antioxidant activity in a cup-of-tea, relative to a positive control for FRAP and to a negative control (ultrapure water) for CCA and ICA.

2.9. Statistical analysis

Results were expressed as mean ± standard deviation (SD) and experiments were conducted at least in triplicate. Significant differences ($p < 0.05$) were assessed by one-way analysis of variance (ANOVA) or Kruskal Wallis one-way analysis of variance on ranks when parametricity of data did not prevail. If significant, the pairwise multiple comparison tests Tukey or Dunn's were applied. Statistical analyses were performed using XLStat2014®.

3. Results and discussion

3.1. Phytochemical analysis

Herbal teas are important sources of polyphenolics in human diet as these compounds are among the most widely occurring secondary metabolites in plants (Balasundram et al., 2006). However, characterization of phenolic groups is difficult due to the different polyphenolic mixtures in each plant. In this sense, fast-screening spectrophotometric methods are the widespread approach when assessing total phenolic or phenolic-groups content in plant extracts (Dai and Mumper, 2010). Tisanes from *C. maritimum* were assessed spectrophotometrically for their total contents in different phenolic groups and results are summarized on Table 1. *Aspalathus linearis* (rooibos) herbal tea was used as comparison because it is also a tisane and is a greatly consumed tea beverage. Rooibos tisanes had higher TPC than sea fennel's samples, which could be expected since the former is reported as rich in polyphenolic compounds (McKay and Blumberg, 2007). The CTC of rooibos samples was also higher than in those of sea fennel, but since rooibos is known as a low tannin tea compared to green or black teas (from *C. sinensis*; Joubert and de Beer, 2011), sea fennel's infusions and decoctions can be considered of comparatively low tannin content. Nevertheless, TFC, HAD and flavonols contents in rooibos tisanes was similar or even lower than those from sea fennel's leaves extracts. Amongst the sea fennel's organs, leaves' infusions and decoctions had the highest levels of all phenolic groups analysed except CTC, followed by flowers tisanes and, lastly, the stems' extracts with the lowest content. CTC was equally low in all of sea fennel tisanes (0.0–0.96 mg/cup-of-tea), which can be deemed positive in terms of flavouring since these compounds are associated with an astringent and unpleasant taste. Working with the same species Houta et al. (2011) also assessed the phenolic contents between different organs but reported higher TPC, TFC and CTC in stems rather than in leaves or flowers; however, those authors used methanolic extracts and studies have already showed that solvent and extraction method can greatly influence results (Buhmann and Papenbrock, 2013). Houta et al. (2011) reported TPC in stems, leaves and flowers between 9 and 14 mg GAE/g dry extract and Meot-Duros and Magné (2009) reported it in bulk aerial organs' methanolic extracts between 10 and 33 mg GAE/g dry extract; they considered that sea fennel had relatively high phenolic content when compared to other crop species as for example spinach and broccoli. These values are lower than those measured in the

Table 1
Yields (g extract/200 mL) and phenolic contents (mg/cup-of-tea) in infusions and decoctions from stems, leaves and flowers of *C. maritimum* and from *A. linearis* (rooibos).

Plant	Organ	Extract	Yields	TPC[a]	TFC[b]	CTC[c]	HAD[d]	Flavonols[e]
C. maritimum	Stems	Infusion	0.302	12.4 ± 0.36^{e}	22.9 ± 0.69^{e}	0.0^{c}	8.97 ± 0.58^{f}	4.60 ± 0.40^{e}
		Decoction	0.319	12.8 ± 0.92^{e}	27.1 ± 1.15^{e}	0.63 ± 0.15^{c}	11.2 ± 0.56^{e}	5.96 ± 0.48^{d}
	Leaves	Infusion	0.478	33.7 ± 0.91^{c}	54.4 ± 6.67^{bc}	0.96 ± 0.25^{c}	25.3 ± 0.67^{a}	15.7 ± 0.44^{a}
		Decoction	0.500	35.3 ± 2.98^{c}	57.2 ± 6.42^{b}	0.63 ± 0.15^{c}	25.2 ± 1.44^{a}	16.2 ± 0.99^{a}
	Flowers	Infusion	0.404	21.2 ± 0.17^{d}	48.0 ± 2.07^{cd}	0.26 ± 0.00^{c}	21.1 ± 0.71^{b}	10.2 ± 0.41^{c}
		Decoction	0.389	22.6 ± 0.99^{d}	40.7 ± 3.55^{d}	0.0^{c}	18.6 ± 1.27^{c}	9.46 ± 0.75^{c}
A. linearis		Infusion		43.1 ± 3.39^{b}	52.7 ± 5.41^{bc}	11.8 ± 2.82^{b}	12.0 ± 0.75^{e}	12.7 ± 0.60^{b}
		Decoction		51.3 ± 1.08^{a}	66.7 ± 2.81^{a}	13.2 ± 1.37^{a}	14.3 ± 0.25^{d}	15.5 ± 0.45^{a}

Data represent the mean ± SD ($n \geq 6$). In each column, different letters mean significant differences ($p < 0.05$).
[a] TPC: total polyphenol content, mg GAE/200 mL, GAE: gallic acid equivalents.
[b] TFC total flavonoid content; mg RE/200 mL, RE: rutin equivalents.
[c] CTC: condensed tannin content, mg CE/200 mL, CE: catechin equivalents.
[d] HAD hydroxycinnamic acid derivatives, mg CAE/200 mL, CAE: caffeic acid equivalent.
[e] mg QE/200 mL, QE: quercetin equivalents.

present study when considering the extraction yields obtained and the TPC per cup-of-tea of sea fennel's organs (see Table 1). Nevertheless, it should be mentioned that phytochemical content can vary according to species provenance, as confirmed by Jallali et al. (2014), since intra-species variables affect biosynthesis of secondary metabolites in plants. Hence, *C. maritimum*'s herbal teas can be considered of comparatively good polyphenolic content, particularly leaves' tisanes, and a potentially good source of these bioactive phytochemicals.

The phenolic profile of infusions and decoctions from the organs of sea fennel was further investigated by HPLC–DAD aiming to identify the individual phenolic compounds. Results (mg/200 mL, i.e., mg/cup-of-tea, calculated based on the extraction yields) are presented in Table 2 and Fig. 1. Ten polyphenolic compounds were identified and quantified in the sea fennel beverages from which *p*-hydroxybenzoic acid, ferulic acid, epicatechin, pyrocatechol and 4-hydroxybenzaldehyde are, to the best of our knowledge, here firstly described in *C. maritimum*. Amid the sea fennel's organs, leaves' herbal teas had consistently higher levels of all the phenolics detected, which is in agreement with the leaves' highest values of phenolic groups (TPC, TFC, HAD, flavonols, Table 1). Chlorogenic acid (CGA) was the dominant phenolic compound in all extracts, reaching more than 8 mg/cup-of-tea in leaves' tisanes and around 3.5 and 2.5 mg/cup-of-tea in flowers and stems samples, respectively. Other reports also found CGA as the major phenolic in sea fennel extracts (Meot-Duros and Magné, 2009; Nabet et al., 2016; Siracusa et al., 2011), and associate its high levels to an antioxidant protection against the oxidative stress endured by plants exposed to such stressful environments (Meot-Duros and Magné, 2009). Those values correspond to 8–17 mg CGA/g extract dw (dry weight) considering the extraction yields (Table 1) and are within the range determined by Meot-Duros and Magné (2009). According to these authors, the sea fennel is among the highest CGA-containing species within the Apiaceae family; accordingly, decoctions from common fennel *Foeniculum vulgare* had lower CGA content (4.54 mg/g; Caleja et al., 2015) than that presently determined in sea fennel's herbal teas. Hence, sea fennel's water extracts, especially from leaves, can be a valuable alternative source of CGA for the food industry. According to Santana-Gálvez et al. (2017) this phytochemical is a promising nutraceutical and food additive attending to its multifunctional properties. In fact, CGA has several reported biological activities including antioxidant, antimicrobial and anti-carcinogenic along with hypoglycaemic, hypolipidaemic and hypotensive properties (Meng et al., 2013; Onakpoya et al., 2015; Santana-Gálvez et al., 2017).

Other main compounds determined in sea fennel's beverages (>1 mg/cup-of-tea; Table 2) were neochlorogenic acid (NCGA) and cryptochlorogenic acid (CCGA) in leaves' tisanes; they were also

Table 2
HPLC–DAD analysis of the phenolic profile (mg/cup-of-tea) of infusions and decoctions from stems, leaves and flowers of *C. maritimum*.

Peak n°	RT (min)	Compound (Peak)	Stems		Leaves		Flowers	
			Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
Phenolic acids								
Hydroxybenzoic acids								
1	1.5	Gallic acid	–	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	4.4	*p*-Hydroxybenzoic acid	0.06	0.06	0.15	0.17	0.04	0.05
Hydroxycinnamic acids								
3	2.8	Neochlorogenic acid	0.45	0.53	1.47	1.73	0.34	0.43
4	7.4	Cryptochlorogenic acid	0.53	0.63	1.59	1.83	0.50	0.64
5	7.8	Chlorogenic acid	2.43	2.42	8.24	8.67	3.33	3.66
6	11.6	Coumaric acid	0.09	0.12	0.36	0.38	0.29	0.31
7	13.0	Ferulic acid	0.15	0.18	0.57	0.77	0.23	0.28
Flavonoids								
Flavanols								
8	10.5	Epicatechin	–	0.35	0.84	1.16	0.26	0.35
Other polyphenols								
9	2.5	Pyrocatechol	0.10	0.10	0.31	0.33	0.08	0.08
10	5.0	4-Hydroxybenzaldehyde	0.01	0.01	0.05	0.06	<LOQ	<LOQ
		TOTAL	**3.82**	**4.41**	**13.57**	**15.12**	**5.06**	**5.80**

RT – retention times; LOQ = 0.01 mg compound/g extract dw.

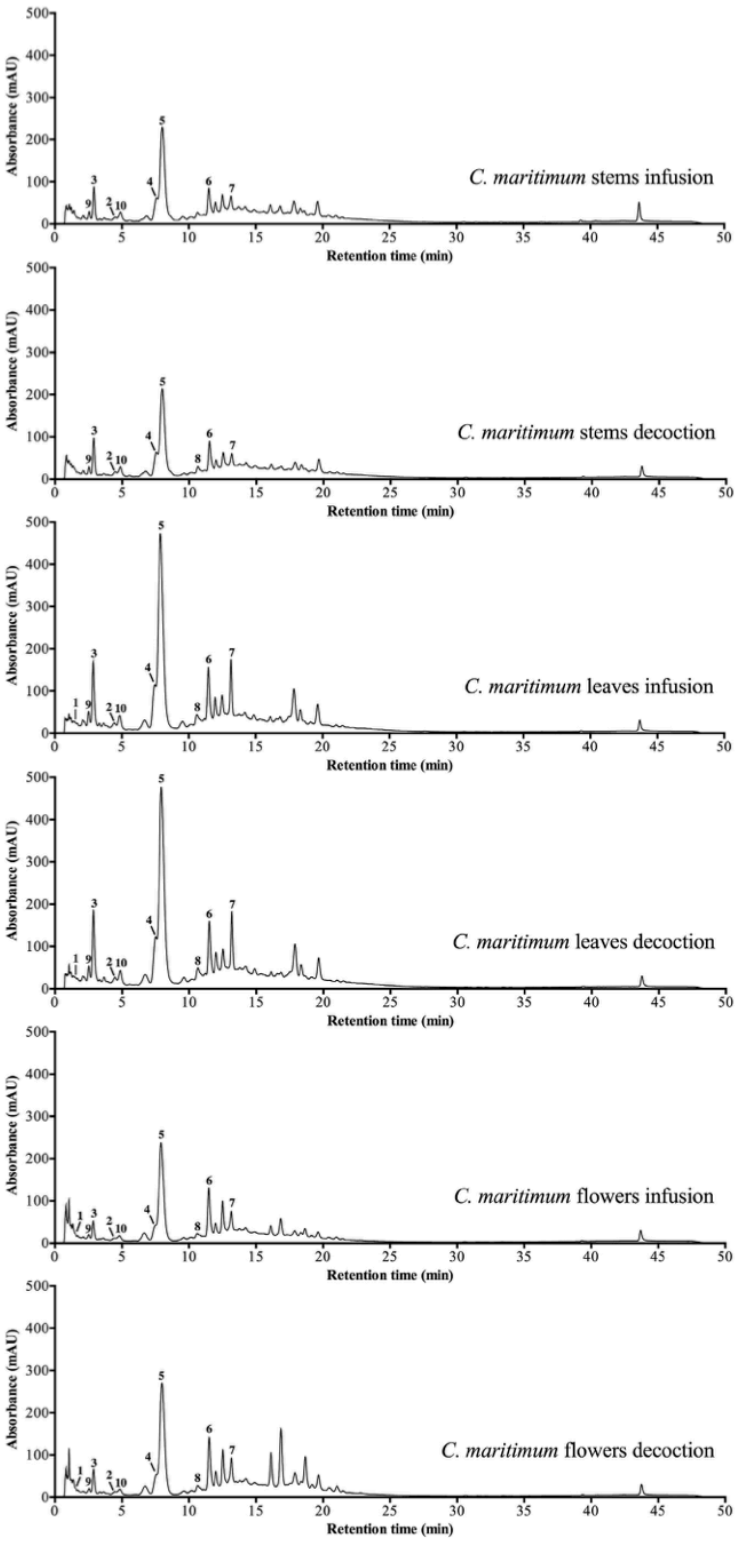

Fig. 1. HPLC–DAD analysis (280 nm) of phenolic compounds in infusions and decoctions from *C. maritimum* organs. Peak numbers refer to the compounds in Table 2.

preferentially detected in stems and flowers' extracts (0.3–0.6 mg/cup-of-tea). NCGA and various other caffeoylquinic acids have already been identified in sea fennel extracts at analogous concentrations (Nabet et al., 2016; Siracusa et al., 2011). They are associated with the strong antioxidant activity of several vegetables (Shahidi and Ambigaipalan, 2015) and with some health promoting effects, as for example the modulation of glucose and lipid metabolism and reduction of blood pressure (Onakpoya et al., 2015). Epicatechin was also a main compound in leaves' tisanes (0.8–1.2 mg/cup-of-tea) and determined in the other samples at lower concentrations (0.2–0.3 mg/cup-of-tea, not detected in stems' infusion). This flavanol was not found reported in literature for sea fennel but common fennel's decoction showed lower values (0.43 mg/g; Caleja et al., 2015). Epicatechin has various beneficial properties described such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic and cardio-protective, among others (Shay et al., 2015). Also preferentially detected in sea fennel's herbal teas was ferulic acid (FA) in leaves' tisanes (0.6–0.8 mg/cup-of-tea), determined in lower amounts in roots and flowers' extracts (0.2–0.3 mg/cup-of-tea). This phenolic acid was also not found described in literature for sea fennel nor was it detected in common fennel's decoction (Caleja et al., 2015); however, FA in *F. vulgare* methanolic extracts represented 3.5% of total phenolics (Roby et al., 2013), a lower ratio than that presently found (4.3–5.1%). Besides is potent antioxidant activity, FA has many recognized bioactivities among which anti-diabetic, anti-inflammatory, anti-carcinogenic and cardio-protective (Kumar and Pruthi, 2014). Other phenolics detected at noteworthy concentrations (0.3–0.4 mg/cup-of-tea) were coumaric acid (CA) in leaves and flowers' samples and pyrocatechol in leaves' herbal teas. CA has been detected in sea fennel but not quantified (Jallali et al., 2012) while pyrocatechol was not found described in literature for this species. The remaining phenolics were detected in minor amounts or below the quantitation limit (LOQ, Table 2). Overall, the phenolics identified in sea fennel's extracts, described in literature as interesting natural bioactive compounds, help explain some of the plant's medicinal uses and highlight the potential use of sea fennel infusions and decoctions as a source of bioactive molecules/products, namely beverages with health promoting potential.

Despite being important sources of polyphenolic compounds (Balasundram et al., 2006), herbal teas can also be an excellent source of other components such as minerals (Ajuwon et al., 2015; Pohl et al., 2016). In this sense, tisanes from sea fennel's organs were analysed for mineral content and results are summarized on Table 3. Sodium was the most abundant element in sea fennel's beverages (19.8–49.3 mg/cup-of-tea), particularly in those from leaves, being higher than the values detected in several herbal teas from commonly used plants used, such as *Cymbopogon citratus* (DC.) Stapf. (lemongrass), *Matricaria chamomilla* L. (chamomile) or *Zingiber officinale* Roscoe (ginger) (Pohl et al., 2016). Na is an essential nutrient, however its recommended daily intake should not exceed 2000 mg (WHO, 2012a). Considering that a cup-of-tea from sea fennel's organs contains no more than 49 mg of Na it is a reasonably safe beverage to include in a daily diet. The other macro-elements were also rather abundant in sea fennel's tisanes with levels of Ca (2.93–19.9 mg/cup-of-tea) and Mg (1.69–5.55 mg/cup-of-tea) being higher in leaves' extracts and K values (2.18–22.6 mg/cup-of-tea) higher in flowers' samples. Among microelements, the sea fennel's beverages had similar values of Fe (11.0–27.3 μg/cup-of-tea), Mn (2.53–17.4 μg/cup-of-tea) and Zn (11.3–35.9 μg/cup-of-tea), higher in leaves' decoction for Fe and Mn. According to a compilation of minerals in tisanes from numerous plants, herbal beverages can be deemed good sources of many elements like Na, Ca, K, Mg, Fe, Mn and Zn (Pohl et al., 2016). Of the adult-daily recommended intakes for Ca (1000–1300 mg/day), Mg (190–260 mg/day), K (3510 mg/day), Fe (9.1–58.5 mg/day), Zn (3–14 mg/day) (WHO/FAO, 2004; WHO, 2012b) and Mn (5–5.5 mg/day; NHMRC, 2006) a cup-of-tea from sea fennel's organs can supply between 0.2%–2% of Ca, 0.7%–2.9% of Mg, 0.06%–0.6% of K, 0.02%–0.3% of Fe, 0.08%–1.2% of Zn and 0.05%–0.3% of Mn. In this sense sea fennel infusions and decoctions may be considered a mineral supplementary source, just like most herbal teas usually are (Pohl et al., 2016). Moreover, the levels of potentially toxic minerals like Cu, Cr, Ni, Pb and Cd, when detected, were below legislated values for plants (Pb: 0.3 mg/kg wet weight; Cd: 0.2 mg/kg wet weight; maximum levels in finished herbal products are not regulated) pointing to the safe consumption of sea fennel's tisanes (EC Regulation, 1881/2006).

3.2. Toxicological evaluation

The toxicity of plant extracts, herbal beverages in particular, must be determined if its safety for human consumption is to be established. Preliminary toxicity screenings are usually assessed through *in vitro* models using different mammalian cell lines to test for cytotoxicity, which delivers quick and reliable results and reduces *in vivo* testing (Rodrigues et al., 2016; Saad et al., 2006). In this study, *C. maritimum*'s tisanes were subjected to a preliminary toxicological evaluation using four different mammalian cell lines, together with *A. linearis* extracts for comparison, and cellular viability is presented in Fig. 2. Sea fennel's extracts had low toxicity with cell viability values always higher than 90% for all cell lines. Rooibos samples had moderate toxicity towards non-tumoural cells (N9 and S17), with viability values (57%–66%) lower than those obtained for the sea fennel beverages (>90%). Samples from both species did not exhibited toxicity against tumoural cells SH-SY5Y and HepG2, since cellular viabilities after applying sea fennel's and rooibos extracts were similar or higher than 90%. As a preliminary toxicological assessment, these results suggest that sea fennel's infusions and decoctions can be considered as non-toxic beverages especially when compared to those obtained for the commercial rooibos tisanes. To the best of our knowledge no toxicological studies of sea fennel extracts are reported but this plant's large use for nutritional and culinary purposes points to its safe consumption (Atia et al., 2011; Renna and Gonnella, 2012).

3.3. Biological activities: in vitro antioxidant properties

Nowadays, natural antioxidants such as food products and/or herbal beverages are in high demand in the market (Sindhi et al., 2013). In fact, consumers are aware of the potential benefits of natural products and are willing to spend more on nutrition and supplements. In this work, the antioxidant potential of "cups-of-tea" from sea fennel's organs was assessed by eight methods targeting radical scavenging activity (RSA) and metal-related potential (Table 4). Results were compared with those obtained with rooibos herbal tea which has well documented antioxidant properties (Ajuwon et al., 2015; Joubert and de Beer, 2011). All extracts from sea fennel were more active against the hydroxyl radical ($OH^{\cdot}$; 44.1–54.4%) than rooibos herbal teas (18.8–25.6%) and all except roots infusions had the same RSA towards DPPH (83.5–88.0% activity) as the rooibos samples (84.6%). The NO scavenging capacity of the sea fennel's decoctions from leaves and flowers (58.6% and 57.5%) also matched rooibos tisane's activity (58.6–59.5%) and samples from sea fennel's leaves (86.6–88.0%) were as effective against the ABTS radical as rooibos extracts (92.7–92.9%). Flowers tisanes were slightly less active than those from leaves towards NO but still had around 80% activity. Moreover, herbal teas from sea fennel's leaves and flowers matched the rooibos beverage capacity to reduce iron (FRAP; 96.1–100%) and matched or surpassed its

Table 3
Mineral content of infusions and decoctions (mg or μg/cup-of-tea) from stems, leaves and flowers of *C. maritimum*.

	Mineral	Stems		Leaves		Flowers	
		Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
Essential elements	Macro-elements (mg/cup-of-tea)						
	Na	33.3 ± 0.32^{b}	32.7 ± 2.80^{b}	40.1 ± 5.44^{ab}	49.3 ± 0.20^{a}	21.9 ± 1.00^{c}	19.8 ± 0.35^{c}
	Ca	2.93 ± 0.01^{b}	3.10 ± 0.14^{b}	17.8 ± 1.93^{a}	19.9 ± 0.51^{a}	4.13 ± 0.15^{b}	4.11 ± 0.19^{b}
	K	3.40 ± 0.18^{cd}	3.64 ± 0.02^{c}	2.18 ± 0.25^{d}	4.58 ± 0.46^{c}	22.6 ± 0.26^{a}	19.3 ± 0.60^{b}
	Mg	1.69 ± 0.03^{c}	1.81 ± 0.00^{bc}	5.03 ± 0.40^{a}	5.55 ± 0.08^{a}	2.40 ± 0.03^{b}	2.41 ± 0.04^{b}
	Micro and trace-elements (μg/cup-of-tea)						
	Fe	18.6 ± 0.78^{b}	15.6 ± 3.12^{bc}	18.9 ± 2.30^{b}	27.3 ± 0.41^{a}	11.0 ± 0.49^{c}	16.0 ± 2.25^{bc}
	Mn	2.62 ± 0.01^{d}	2.53 ± 0.12^{d}	12.7 ± 0.17^{b}	17.4 ± 0.38^{a}	4.00 ± 0.23^{c}	4.28 ± 0.07^{c}
	Zn	35.9 ± 1.46^{a}	17.4 ± 9.57^{a}	12.3 ± 0.20^{a}	11.3 ± 0.27^{a}	12.8 ± 0.74^{a}	28.3 ± 17.0^{a}
	Cu	5.00 ± 0.15^{ab}	3.81 ± 1.00^{ab}	3.39 ± 0.24^{b}	5.47 ± 0.11^{a}	4.26 ± 0.13^{ab}	4.02 ± 0.02^{ab}
	Cr	0.16 ± 0.07^{a}	0.20 ± 0.00^{a}	0.61 ± 0.49^{a}	0.49 ± 0.12^{a}	0.27 ± 0.16^{a}	0.22 ± 0.02^{a}
	Ni	< LOD	< LOD	< LOD	0.44 ± 0.00	< LOD	< LOD
Non-essential elements	Pb	< LOD	< LOD	< LOD	2.66 ± 0.00	< LOD	< LOD
	Cd	0.52 ± 0.00^{a}	< LOD	0.22 ± 0.00^{a}	< LOD	< LOD	< LOD

Data represent the mean ± SD ($n = 3$). In each row different letters mean significant differences ($p < 0.05$).
LOD: Cd, 0.08 μg/cup-of-tea; Ni, 0.06 μg/cup-of-tea; Pb, 0.15 μg/cup-of-tea.

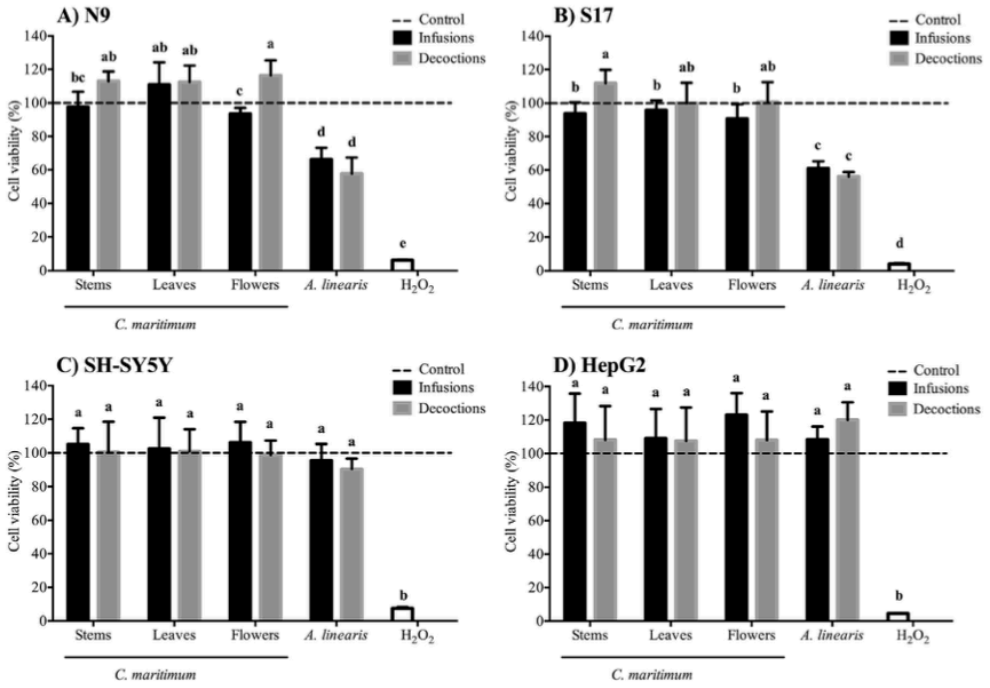

Fig. 2. Toxicity of infusions and decoctions, applied at the concentration of 100 μg/mL (extract dw) from *C. maritimum* organs and *A. linearis* (rooibos) on mammalian cell lines: A) N9, B) S17, C) SH-SY5Y and D) HepG2. Cells treated only with cell culture medium were used as controls; H_2O_2 was used as positive control for cell toxicity. Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each graph different letters mean significant differences ($p < 0.05$).

copper chelating activity, although being moderate (30.7–38.2%). The capacity to chelate iron, while also moderate, was similar between sea fennel's stems and flowers infusions (36.0–36-5%) and rooibos decoction (41.8%), and between the remaining sea fennel's extracts and rooibos infusion. Cups-of-tea from sea fennel's organs had approximately 80% of capacity to scavenge the superoxide radical ($O_2^{\cdot-}$), although they were less effective than rooibos (aprox. 84%). Furthermore, although less active in metal-related potential, sea fennel and rooibos extracts were at least as efficient as the positive controls in radical-scavenging activity.

As can be deduced from our results, the antioxidant capacity of the sea fennel's herbal teas from leaves and flowers were overall as effective as those of rooibos tisanes. Additionally, among the sea fennel's organs, leaves and flowers extracts had the highest scavenging capacity, FRAP and copper chelating activity. Similarly, Houta et al. (2011) reported a higher scavenging activity towards DPPH in sea fennel methanolic extracts from leaves, followed by flowers and stems with the lowest RSA. Nevertheless, high

Table 4
Radical scavenging on DPPH, ABTS, NO, $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals, ferric reducing antioxidant power (FRAP) and metal-chelating activities on copper (CCA) and iron (ICA) of infusions and decoctions from stems, leaves and flowers of *C. maritimum* and *A. linearis* (rooibos). Results are expressed as antioxidant activity (% activity in a cup-of-tea).

Plant/compound	Organ	Extract	Antioxidant activity (%)							
			DPPH	NO	ABTS	$O_2^{\cdot-}$	$OH^{\cdot}$	FRAP	CCA	ICA
C. maritimum	Stems	Infusion	79.4 ± 5.28^{c}	45.1 ± 0.69^{d}	62.1 ± 7.83^{d}	52.1 ± 4.82^{c}	46.1 ± 5.11^{bc}	88.3 ± 0.67^{c}	25.6 ± 2.46^{de}	36.0 ± 4.79^{bcd}
		Decoction	83.5 ± 1.43^{abc}	54.5 ± 2.22^{c}	71.3 ± 4.7^{c}	55.7 ± 3.23^{c}	54.4 ± 4.49^{b}	85.4 ± 1.28^{c}	22.0 ± 3.64^{e}	17.7 ± 3.96^{f}
	Leaves	Infusion	86.5 ± 0.95^{a}	37.0 ± 1.44^{e}	88.0 ± 2.97^{ab}	76.9 ± 1.46^{b}	44.1 ± 5.05^{c}	98.6 ± 3.40^{ab}	34.0 ± 2.25^{bc}	31.1 ± 3.87^{cde}
		Decoction	86.0 ± 4.34^{ab}	58.6 ± 1.07^{b}	86.6 ± 3.67^{ab}	76.6 ± 1.15^{b}	48.9 ± 4.39^{bc}	98.8 ± 2.99^{ab}	38.2 ± 2.69^{b}	26.0 ± 7.67^{ef}
	Flowers	Infusion	88.0 ± 0.16^{a}	15.1 ± 1.12^{f}	80.3 ± 6.23^{b}	78.2 ± 1.51^{b}	48.0 ± 4.69^{bc}	96.1 ± 0.81^{b}	37.8 ± 3.51^{b}	36.5 ± 5.51^{bc}
		Decoction	87.3 ± 0.98^{a}	57.5 ± 0.77^{b}	82.6 ± 6.03^{b}	77.5 ± 0.87^{b}	53.2 ± 3.39^{b}	100 ± 0.00^{a}	30.7 ± 2.86^{cd}	30.1 ± 6.17^{cde}
A. linearis		Infusion	84.6 ± 0.41^{ab}	58.6 ± 0.69^{b}	92.7 ± 0.85^{a}	84.4 ± 0.24^{a}	18.8 ± 2.40^{d}	98.1 ± 2.00^{ab}	26.6 ± 3.52^{de}	26.2 ± 1.70^{def}
		Decoction	84.6 ± 0.51^{ab}	59.5 ± 1.29^{b}	92.9 ± 0.63^{a}	84.9 ± 0.15^{a}	25.6 ± 1.40^{d}	100 ± 0.00^{a}	28.6 ± 3.73^{cd}	41.8 ± 4.29^{b}
BHT[a]			81.7 ± 1.65^{bc}		93.4 ± 0.26^{a}			–		
Ascorbic acid[a]				90.6 ± 1.35^{a}						
Catechin[a]						75.2 ± 2.83^{b}	84.4 ± 9.31^{a}			
EDTA[a]									94.6 ± 0.36^{a}	99.7 ± 0.15^{a}

Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9). In each column different letters mean significant differences ($p < 0.05$).
[a] Positive controls tested at 1 mg/mL (BHT, catechin and EDTA) or 10 mg/mL (ascorbic acid).

antioxidant activity has already been described in sea fennel undifferentiated aerial extracts (Houta et al., 2011; Jallali et al., 2014; Meot-Duros and Magné, 2009; Romojaro et al., 2013; Siracusa et al., 2011). Our results confirm the sea fennel's *in vitro* antioxidant potential, mostly of its leaves and flowers, and thus show that beverages made from this plant's organs may be useful in preventing oxidative-stress related diseases much like the famous rooibos herbal tea is reported to be (Ajuwon et al., 2015).

The antioxidant activity of plant extracts is closely associated to their phenolic content (Dai and Mumper, 2010) and, in fact, infusions and decoctions from leaves were consistently the extracts with the higher amounts of all phenolic groups except CTC (Table 1), followed by flower's tisanes. This suggests that polyphenols may be the major contributors to the antioxidant capacity of these sea fennel's extracts, an association confirmed by numerous previous studies attesting to the phenolics role as antioxidants in plants, particularly in halophytes (Ksouri et al., 2012). In fact, the environmental stress factors that salt-tolerant species like sea fennel endure influences their phenolic content and related antioxidant activity (Buhmann and Papenbrock, 2013). Moreover, the amount of individual phenolics (Table 2) can also contribute to the stronger antioxidant activities in leaves and flowers tisanes since most of these phytochemicals were determined in higher amounts in these organ's beverages. For example, the main component detected, chlorogenic acid, is an antioxidant compound (Meng et al., 2013) already linked to the sea fennel's strong radical scavenging ability (Meot-Duros and Magné, 2009). Some of the other phenolics determined in higher amounts in leaves and flowers tisanes may have also contributed through addictive and/or synergistic effects, namely NCGA and CCGA, which are known antioxidants (Shahidi and Ambigaipalan, 2015). Additionally, sea fennel's leaves beverages had higher levels of all the phenolics detected, which can account for the slightly higher ABTS radical scavenging activity of these tisanes.

Phenolics are recognized powerful antioxidants and plant-products like herbal teas are an important dietary source of these phytochemicals (Gruenwald, 2009; Ksouri et al., 2012). The intake of antioxidants is associated with the prevention or amelioration of oxidative stress-related diseases, as for example neurodegenerative disorders, cardiovascular dysfunction, diabetes and cancer, and their consumption has become a strategy to address such health challenges (Sindhi et al., 2013; Lu and Yen, 2015). Thus, the estimated antioxidant capacity and phytochemical contents of the extracts from sea fennel's organs suggest that sea fennel's herbal teas, particularly from leaves and flowers, can be an alternative source for natural antioxidants with possible health benefits beyond its nutritional role in terms of minerals. Herbal teas are popular beverages consumed for their pleasant taste and therapeutic properties (Pohl et al., 2016) and the use of *C. maritimum* as an herbal beverage may have commercial potential. It could well follow the example of *A. linearis*: the rooibos plant had no commercial value until its potential was recognized and nowadays it is highly valued in the food industry and a worldwide consumed herbal tea (Joubert and de Beer, 2011).

4. Conclusion

From our results it is clear that infusions and decoctions made from *C. maritimum* leaves and flowers have a high polyphenolic content, a strong antioxidant potential, an interesting mineral profile and can be considered as non-toxic beverages in view of the preliminary toxicological assessment with *in vitro* models. Thus, sea fennel's leaves and flowers herbal teas could be a potential source of bioactive molecules and/or products for the food industry, as for example antioxidants and minerals.

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CHAPTER 5

CHEMICAL PROFILING OF INFUSIONS AND DECOCTIONS OF *HELICHRYSUM ITALICUM* SUBSP. *PICARDII* BY UHPLC-PDA-MS AND *IN VITRO* BIOLOGICAL ACTIVITIES COMPARATIVELY WITH GREEN TEA (*CAMELLIA SINENSIS*) AND ROOIBOS TISANE (*ASPALATHUS LINEARIS*)

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ABSTRACT

Several medicinal plants are currently used by the food industry as functional additives, for example botanical extracts in herbal drinks. Moreover, the scientific community has recently begun focusing on halophytes as sources of functional beverages. *Helichrysum italicum* subsp. *picardii* (everlasting) is an aromatic halophyte common in southern Europe frequently used as spice and in traditional medicine. In this context, this work explored for the first time *H. italicum* subsp. *picardii* as a potential source of innovative herbal beverages with potential health promoting properties. For that purpose, infusions and decoctions were prepared from roots, vegetative aerial-organs (stems and leaves) and flowers and evaluated for *in vitro* antioxidant and anti-diabetic activities. Samples were also assessed for toxicity in different mammalian cell lines and chemically characterized by spectrophotometric methods and ultra-high performance liquid chromatography–photodiode array–mass-spectrometry (UHPLC-PDA-MS). Results were expressed relating to 'a cup-of-tea' and compared with those obtained with green tea (Camellia sinensis) and rooibos tisane (Aspalathus linearis). Tisanes from the everlasting's above-ground organs, particularly flowers, have high polyphenolic content and several phenolics were identified; the main compounds were chlorogenic and quinic acids, dicaffeoylquinic-acid isomers and gnaphaliin-A. The antioxidant activity of beverages from the everlasting's above-ground organs matched or surpassed that of green tea and rooibos. Its anti-diabetic activity was moderate and toxicity low. Overall, our results suggest that the everlasting is a potential source of innovative and functional herbal beverages.

1. Introduction

Tea is one of the most common beverages in the world. Pairing a pleasant taste to stimulating effects and potential health benefits, this popular drink is a cocktail of biologically active phytochemicals as, for example, catechins and gallocatechins. Herbal teas, or tisanes, are infusions or decoctions of any plant material whereas real teas are prepared from the leaves of the tea plant, *Camellia sinensis* (L.) Kuntze. [1]. The health benefits derived from the consumption of real tea, particularly the green type, are well described and include cancer prevention, reduction of cardiovascular risk, anti-diabetic and anti-obesity properties and/or protection against oxidative damage and oxidative stress-related diseases [1,2]. As for herbal teas, consumption benefits can be associated with the plants' medicinal properties. For example, the popular herbal red tea, from the rooibos plant *Aspalathus linearis* (Burm.f.) Dahlg., is marketed for its high antioxidant and anti-ageing potential [3].

Nowadays, a wide panoply of medicinal plants (e.g. *Aloe vera* and *Hibiscus* sp.) are used by the food industry as sources of functional additives, such as botanical extracts in herbal beverages, and are commercially available in local stores and supermarkets to be

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consumed as tisanes for health-related purposes [4]. But medicinal extremophiles, halophytes in particular, although representing an outstanding reservoir of bioactive compounds are still quite unexploited [5]. Nevertheless, the scientific community has just recently begun focusing on aqueous extracts from halophytes with potential to be functional beverages, like *Limonium algarvense* Erben [6] and *Crithmum maritimum* L. [7]. Moreover, specialty stores have started to sell some halophytes as functional herbal beverages, namely sea buckthorn (*Hippophae rhamnoides*), and the gourmet food market has also turned its attention to halophyte products, like *Salicornia* sp. [8,9].

Helichrysum plants (Asteraceae), commonly called "everlasting", have medicinal uses reported since the first centuries, its decoctions being referred to as diuretic, or used to treat urinary disorders, burns, venomous bites or hernias [10]. *Helichrysum italicum* (Roth) G. Don plays an important role in the traditional medicine of Mediterranean countries and is often used as spice due to its curry-like scent. Its subspecies *H. italicum* (Roth) G. Don subsp. *picardii* (Boiss & Reuter) Franco is a facultative halophyte found in the southern Europe, including Portugal [10,11]. Folk therapeutic uses of infusions and decoctions of the plant are associated to analgesic properties and dermatologic, respiratory and digestive disorders with inflammatory, allergic or infectious components [10,12]. Research concerning *H. italicum* focuses mainly on organic extracts and *in vitro* studies indicate that this everlasting has antimicrobial and anti-inflammatory properties, among others, and contains a wide phytochemical profile that includes different classes of bioactive molecules from which the most common are phenolic compounds and terpenes [10,12,13].

Herbal teas are a major source of dietary bioactive phytochemicals in our diet, including phenolics with recognized antioxidant properties and with beneficial outcomes in certain health challenges [4,14]. Oxidative stress is an underlying cause for several degenerative diseases and the use of antioxidants can prevent or reduce the severity of oxidative stress-related diseases [15]. Moreover, consumption of antioxidants from natural sources has become a consumer-trend for health purposes, promoting the antioxidant market growth [14]. In this sense, medicinal plants like *H. italicum* subsp. *picardii* have a high commercial potential to be explored not only in traditional medicine but also as herbal functional beverages in the health foods category. A similar approach has already been reported for different plants, including glycophytes such as *Lathyrus* species [16] and *Hymenocrater bituminosus* L. [17] and halophytes, like *Chritmum maritimum* L. [7], *Limonium algarvense* L. [6] and *Juncus* species [18].

To the best of our knowledge there is no information regarding the biological activities or phenolic composition of infusions and decoctions of this everlasting species. Therefore, this work aimed to evaluate if this everlasting could be explored as a source of innovative food additives. For that purpose, infusions and decoctions were prepared from roots, vegetative aerial-organs and flowers from the everlasting and evaluated for *in vitro* antioxidant and anti-diabetic activities, and for polyphenolic profile. Additionally, a preliminary toxicological evaluation was made *in vitro* by determining samples toxicity against mammalian cells. Green and herbal red (rooibos) teas were used for comparison since they are the most consumed tea beverages worldwide and are sought for their strong antioxidant properties.

2. Materials and methods

2.1. Reagents

All chemicals used were of analytical grade. Reagents 1,1-diphenyl-2picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), ethylenediamine tetraacetic acid (EDTA), pyrocatechol violet, sodium nitrite, aluminium chloride, butylated hydroxytoluene (BHT), formic acid and ammonium formate were purchased from Sigma-Aldrich (Germany). Ultra-high performance liquid chromatography (UHPLC) grade acetonitrile was purchased from Biosolve (The Netherlands). Merck (Germany) supplied phosphoric acid and Folin-Ciocalteau (F-C) phenol reagent. Commercially available mixtures to calibrate the mass spectrometer, i.e., MSCAL5–1EA (caffeine, tetrapeptide "Met-Arg-Phe-Ala", Ultramark) for positive ion mode and MSCAL6–1EA (sodium dodecylsulfate, taurocholic acid sodium salt, Ultramark) for negative ion mode were purchased from Supelco (USA). Reference standards apigenin, catechin, epicatechin, epigallocatechin, epigallocatechin gallate, flavone, 4-hydroxybenzaldehyde, naringin, quercetin, rutin, uvaol, and caffeic, chlorogenic, coumaric, ferulic, gallic, gentisic, m-hydroxybenzoic, *p*-hydroxybenzoic, oleanolic, rosmarinic, salicylic and syringic acids were purchased from Sigma-Aldrich (Germany); apigenin-7-*O*-glucoside (apigetrin), cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside chloride (ideain chloride), cyanidin-3-*O*-glucoside chloride (kuromanin chloride), cyanidin-3-*O*-rutinoside chloride (keracyanin chloride), (+)-dihydrokaempferol ((+)-aromadendrin), galangin, kaempferol, kaempferol-3-*O*-glucoside (astragalin), luteolin, naringenin, quercetin-3-*O*-arabinoside (avicularin), quercetin-3-*O*-galactoside (hyperin), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rhamnoside (quercitrin), phloretin, phloretin-O-20-glucoside (phloridzin), and procyanidin B2 were purchased from Phytolab (Germany); hesperidin, hesperidin methyl chalcone, limonin, neohesperidin dihydrochalcone, protocatechuic acid, propyl gallate, and sinapinic, dihydrocaffeic, hydroferulic, ellagic, and quinic acids were obtained from Sigma–Aldrich (Belgium). Additional reagents/solvents were obtained from VWR International (Belgium).

2.2. Plant collection

Whole plants of *H. italicum* subsp. *picardii* were collected in the Ria Formosa area, a coastal lagoon in south Portugal, near Cabanas de Tavira (37°07′51.3"N 7°36′35.6"W) in June 2013. The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). A voucher specimen is kept at the herbarium of the Marbiotech laboratory (voucher code MBH32). Plants were divided in roots, vegetative aerial-organs (stems and leaves) and flowers, oven dried for 3 days at 50 °C, milled and stored at −20 °C until use. Dried leaves of green tea plant (*C. sinensis*, produced in Azores, Portugal) and rooibos plant (*A. linearis*, produced in Cape Town, South Africa.) were bought in a regional supermarket, milled and stored at −20 °C.

2.3. Extracts preparation: "cup-of-tea" infusions and decoctions

Extracts were prepared by homogenizing 1 g of the dried plant material in 200 mL of ultrapure water to equal a "cup-of-tea". For infusions biomass was immersed in boiling water for 5 min, for decoctions biomass was boiled in water for 5 min. Aqueous extracts were filtered (Whatman n° 4) and stored at −20 °C until use. Independent extractions ($n \geq 3$) of the different plant parts were made and extracts from the different extractions were tested for their bioactivities and phytochemical (spectrophotometric) content. As no significant differences were found among corresponding extracts from the different extractions, for the LC-PDA-MS analysis aliquots of the extracts were freeze-dried and pooled accordingly,

and stored in a moist free environment at −20 °C protected from light.

2.4. Phytochemical composition of the extracts

2.4.1. Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content

TPC was determined by the F-C assay with absorbance measured at 725 nm using gallic acid as a standard; results were expressed as milligrams of gallic acid equivalents per cup-of-tea (mg GAE/200 mL). TFC was estimated by the aluminium chloride colorimetric method; absorbance was measured at 510 nm using rutin as standard and results were expressed as rutin equivalents per cup-of-tea (mg RE/200 mL). The CTC was assessed by the 4-dimethylaminocinnamaldehyde (DMACA) method; absorbance was measured at 640 nm using catechin as standard and results were expressed as mg of catechin equivalents per cup-of-tea (mg CE/200 mL). All methods are described in Rodrigues et al. [19].

2.4.2. Hydroxycinnamic acid derivatives (HAD) and flavonols content

HAD and flavonols were estimated as described by Rodrigues et al. [19]. Absorbance was read at 320 nm to determine HAD using caffeic acid as standard, and at 360 nm to estimate flavonols using quercetin as standard. Results were expressed as standard equivalents per cup-of-tea (CAE and QE, respectively; mg/200 mL).

2.4.3. Profile of moderately polar compounds by UHPLC

Standard stock solutions were prepared in UHPLC-grade methanol (1 mg/mL) and stored in the dark, 4 °C (standards are listed in Section 2.1. Reagents). Dilutions were prepared in 60:40 (*v*:*v*) methanol:ammonium formate buffer (40 mM). Approximately 15 mg of freeze-dried *H. italicum* subsp. *picardii* pooled extracts were dissolved in 20 mL 60:40 methanol:water + ammonium formate (40 mM) followed by 10 min sonication (40 kHz, 100 W). Samples were centrifuged (3000 rpm), supernatants diluted 100 x and stored together with undiluted extracts at 4 °C until analysis. Both undiluted and 100-fold diluted everlasting extracts were analysed with a generic ultra-high performance liquid chromatography – photodiode array – mass spectrometry (UHPLC-PDA-MS) method for moderately polar phytochemicals adapted from De Paepe et al. [20]. For analysis, 5 μL of extract was injected with a CTC PAL™ autosampler (CTC Analytics, Zwingen, Switzerland) on a Waters Acquity UPLC BEH SHIELD RP18 column (3.0 mm × 150 mm, 1.7 μm; Waters, Milford, MA) and thermostatically (40 °C) eluted with an Accela™ quaternary solvent manager and a 'Hot Pocket' column oven (Thermo Fisher Scientific, Bremen, Germany). The mobile phase solvents consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), and the gradient was set as follows (min/%A): 0.0/100, 9.91/74, 18.51/35, 18.76/0, 23.76/0, 23.88/100, 26.00/100. For detection, an MS (Q Exactive™; Thermo Fisher Scientific, Bremen, Germany) was used with heated electrospray ionization (HESI). For quantitative analysis, full scan data were acquired using polarity switching with a mass/charge (*m*/*z*) range of 120–1800 and resolving power set at 70 000 at full width at half maximum (FWHM). Spray voltage was set at ±2.5 kV, sheath gas and auxiliary gas at 47 and 15 (adimensional) respectively, and capillary temperature at 350 °C. The lowest calibration point that was included in the calibration curve was used to calculate the LOQs. The LOQs can be different for the same compound between extracts as the yields (mg dry extract/g plant value) differ per extract; these yields were used to calculate the LOQs. The concentration ranges described by De Paepe et al. [20] were also used during the present work. As quality control, a midrange calibration point was chosen as continuing calibration verification standard (CCV) in order to verify that the calibration of the analytical system was still acceptable. The frequency of CCV analysis was once every ten injections. Data were also recorded using data dependent fragmentation ($ddMS^2$) in positive and negative ionization mode (one analysis per mode) to obtain additional structural information (resolving power set at 17 500 FWHM, stepped collision energy 10, 30, 50 V, isolation window: 4 *m*/*z*). The PDA detector was set to scan from 190 to 800 nm during all analyses. Results regarding concentrations of identified compounds were calculated as μg/g dried plant material, i.e. μg/cup-of-tea, based on the extracts' yield.

2.5. Antioxidant activity

2.5.1. Determination of antioxidant activity by five radical-based assays

The radical scavenging activities (RSA) on DPPH, nitric oxide (NO), ABTS, superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals was evaluated as described previously [6,19] using respectively BHT, catechin (1 mg/mL) or ascorbic acid (10 mg/mL) as positive controls. Results were expressed as percentage of antioxidant activity in a cup-of-tea, relative to a control containing ultrapure water.

2.5.2. Determination of antioxidant activity by three metal-related methods

The ferric reducing antioxidant power (FRAP) of the extracts, i.e., their ability to reduce Fe^{3+}, along with the metal chelating activities on copper (CCA) and iron (ICA) were assayed as described by Rodrigues et al. [19], using BHT and EDTA as positive controls (1 mg/mL). Results were calculated and expressed as percentage of antioxidant activity in a cup-of-tea, relative to a positive control for FRAP and to a negative control (ultrapure water) for CCA and ICA.

2.6. in vitro anti-diabetic activity: inhibition of α-glucosidase

The microbial α-glucosidase inhibitory activity was determined according to Kwon et al. [21], using acarbose as a positive control (10 mg/mL). The enzyme was obtained from the yeast *Saccharomyces cerevisiae*. Results were expressed as percentage of inhibitory activity in a cup-of-tea, relative to a control (ultrapure water).

2.7. Toxicological evaluation of the samples

Cell culture was made as described by Rodrigues et al. [6]. Murine microglia cell line (N9 cells) was obtained from the Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal); human hepatocellular carcinoma cell line (HepG2 cells) was provided by Dr. Vera Marques, and murine bone marrow stromal cell line (S17 cells) by Dr. Nuno Santos (CBME, University of Algarve, Portugal). Toxicity of the samples was evaluated following Rodrigues et al. [6]. Freeze-dried extracts 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; hydrogen peroxide (H_2O_2) was used as positive control for cell toxicity. Cell viability was determined by the MTT assay and absorbance measured at 590 nm. Results were expressed in terms of cell viability (%).

2.8. Statistical analysis

Results were expressed as mean ± standard deviation (SD), and experiments were conducted at least in triplicate. Significant differences ($p < 0.05$) were assessed by one-way analysis of variance (ANOVA) using the Tukey pairwise multiple comparison test or Kruskal Wallis one-way analysis of variance on ranks (Dunn's test)

Table 1
Phenolic content[1] (mg/cup-of-tea) in infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas: total polyphenol content (TPC), total flavonoid content (TFC) condensed tannin content (CTC), hydroxycinnamic acid derivatives (HAD) and flavonols. In each column, different letters mean significant differences ($p < 0.05$).

Plant	Organ	Extract	TPC (mg GAE/200 mL)	TFC (mg RE/200 mL)	CTC (mg CE/200 mL)	HAD (mg CAE/200 mL)	Flavonols (mg QE/200 mL)
H. italicum subsp. *picardii*	Roots	Infusion	13.9 ± 0.49^{i}	20.3 ± 1.66^{e}	<LQ	13.1 ± 1.16^{e}	8.55 ± 1.13^{f}
		Decoction	20.5 ± 1.45^{h}	26.7 ± 2.77^{e}	<LQ	17.4 ± 0.38^{d}	9.82 ± 0.30^{f}
	Vegetative aerial-organs	Infusion	62.0 ± 0.95^{e}	91.8 ± 9.67^{b}	<LQ	51.3 ± 2.86^{c}	24.8 ± 1.47^{c}
		Decoction	70.2 ± 4.35^{d}	89.0 ± 7.09^{b}	<LQ	56.3 ± 2.54^{b}	26.2 ± 1.18^{c}
	Flowers	Infusion	69.9 ± 3.88^{d}	101 ± 2.55^{b}	<LQ	58.3 ± 2.63^{b}	34.4 ± 1.62^{b}
		Decoction	76.5 ± 2.62^{c}	119 ± 15.6^{a}	<LQ	65.9 ± 1.78^{a}	38.1 ± 1.10^{a}
C. sinensis		Infusion	91.7 ± 2.61^{b}	47.8 ± 2.23^{d}	67.3 ± 5.96^{b}	11.5 ± 1.08^{ef}	9.37 ± 0.83^{f}
		Decoction	107 ± 4.44^{a}	48.8 ± 7.58^{d}	73.7 ± 2.99^{a}	8.76 ± 1.07^{f}	6.12 ± 0.85^{g}
A. linearis		Infusion	43.1 ± 3.39^{g}	52.7 ± 5.41^{d}	11.8 ± 2.82^{c}	12.0 ± 0.75^{ef}	12.7 ± 0.60^{e}
		Decoction	51.3 ± 1.08^{f}	66.7 ± 2.81^{c}	13.2 ± 1.37^{c}	14.3 ± 0.25^{de}	15.5 ± 0.45^{d}

GAE – Gallic acid equivalents; RE – Rutin equivalents; CE – Catechin equivalents; CAE – Caffeic acid equivalents; QE – Quercetin equivalents; LQ – Limit of quantification.
[1] Data represent the mean ± SD ($n \geq 6$). LQ (CTC) = 2.45 mg/200 mL.

Table 2
Concentrations of compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs (μg/g dry biomass, i.e., μg/cup-of-tea), calculated with reference standards using LC–MS. Quantitation limits are presented as ≤ LOQs (μg/g dry biomass).

[a]Peak n°	Compound	[b]RT (min)	Roots		Vegetative aerial-organs		Flowers	
			Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
	Quinic acid	1.56	300	510	8200	8700	4900	4700
	Protocatechuic acid	6.38	2.3	2.3	82	90	41	49
	p-Hydroxybenzoic acid	8.74	≤4	≤3	40	52	48	53
1	Chlorogenic acid	8.94	190	190	6900	7700	6000	5200
	Syringic acid	9.50	≤37	≤31	160	170	≤105	≤135
2	Caffeic acid	9.74	11	21	57	73	150	140
	Rutin	12.10	≤1.4	≤1.2	≤3	≤5	60	60
	Coumaric acid	12.20	≤0.5	≤0.4	6.2	6.9	4.5	6.6
	Ferulic acid	12.36	≤4	5.2	≤10	≤14	20	19
3	Hyperin and/or isoquercitrin	12.62	≤9	≤8	≤23	≤31	350	360
	Apigetrin	13.28	≤2	≤2	36	54	41	74
	Astragalin	13.35	≤2	≤2	29	45	490	600
	Salicylic acid	14.09	≤1.4	≤1.2	14	15	4.9	5.1
	Quercetin	16.44	≤4	≤3	≤10	≤14	12	≤15
	Kaempferol	18.13	≤2	≤2	4.9	6.8	36	45
	Galangin	20.10	≤2	≤2	34	59	41	79
	Oleanolic acid	23.24	≤10	13	≤23	110	≤28	40

[a] Corresponding peak number in the chromatograms on Fig. 1.
[b] T – retention times.

when parametricity of data did not prevail. Statistical analyses were performed using XLStat2014® by Addinsoft (Spain).

3. Results and discussion

3.1. Phytochemical analysis

The phenolic contents of the samples were assessed by spectrophotometric methods, namely the total contents in polyphenols (TPC), flavonoids (TFC) and condensed tannins (TCT), hydroxycinnamic acid derivatives (HAD) and flavonols, and are presented as mg per cup-of-tea (mg/200 mL) in Table 1 (further information pertained to the methods is presented in Table S1, supplementary material). The green tea decoction and infusion had the highest TPC (107 and 91.7 mg/cup-of-tea, respectively), followed by the extracts from flowers and vegetative aerial-organs (stems & leaves) of *H. italicum* subsp. *picardii*, which in turn were richer in TPC than rooibos tisanes. The TFC was greatest in the flowers' decoction from *H. italicum* subsp. *picardii* (119 mg/cup-of-tea) followed by its infusions and the vegetative aerial-organs extracts, showing higher flavonoid content than the green and herbal red teas. The HAD and flavonols showed a similar pattern to the TFC: highest values in the decoction from the everlasting flowers (65.9 and 38.1 mg/cup-of-tea, respectively), and cups-of-tea from flowers and stems & leaves having more of these compounds than green teas and rooibos tisanes. Content of condensed tannins in *H. italicum* subsp. *picardii* teas was below the limit of quantification (2.45 mg/cup-of-tea), which can be considered positive in terms of flavoring from the consumer's perspective given the astringent taste these compounds are known for. Overall, tisanes from *H. italicum* subsp. *picardii* above-ground organs, particularly decoctions from flowers, can be considered of high polyphenolic content especially if compared to the phenolic-rich *C. sinensis* and herbal *A. linearis* teas [22,23].

The phytochemical profile of infusions and decoctions from *H. italicum* subsp. *picardii* organs was further analysed by a generic LC-PDA-MS method for moderately polar phytochemicals, such as phenolic constituents. The analytical LC-PDA-MS methodology, adapted from De Paepe et al. [20], was previously validated by the same authors [20] for the quantitation of phenolic constituents in apple cultivars. The performance characteristics taken into account for the validation of the measurement method were curve fit, range, sensitivity (instrumental detection limit, instrumental quantification limit, method limits of detection and quantification), precision (repeatability, intermediate precision) and trueness, as well as specificity. The goal of the LC-PDA-MS analyses during this study is to explore the phytochemical profile of infusions and decoctions from *H. italicum* subsp. *picardii* organs, *i.e.* to (tentatively) identify unknown phytochemical constituents and to get an estimate of their concentrations (when reference standards were available)

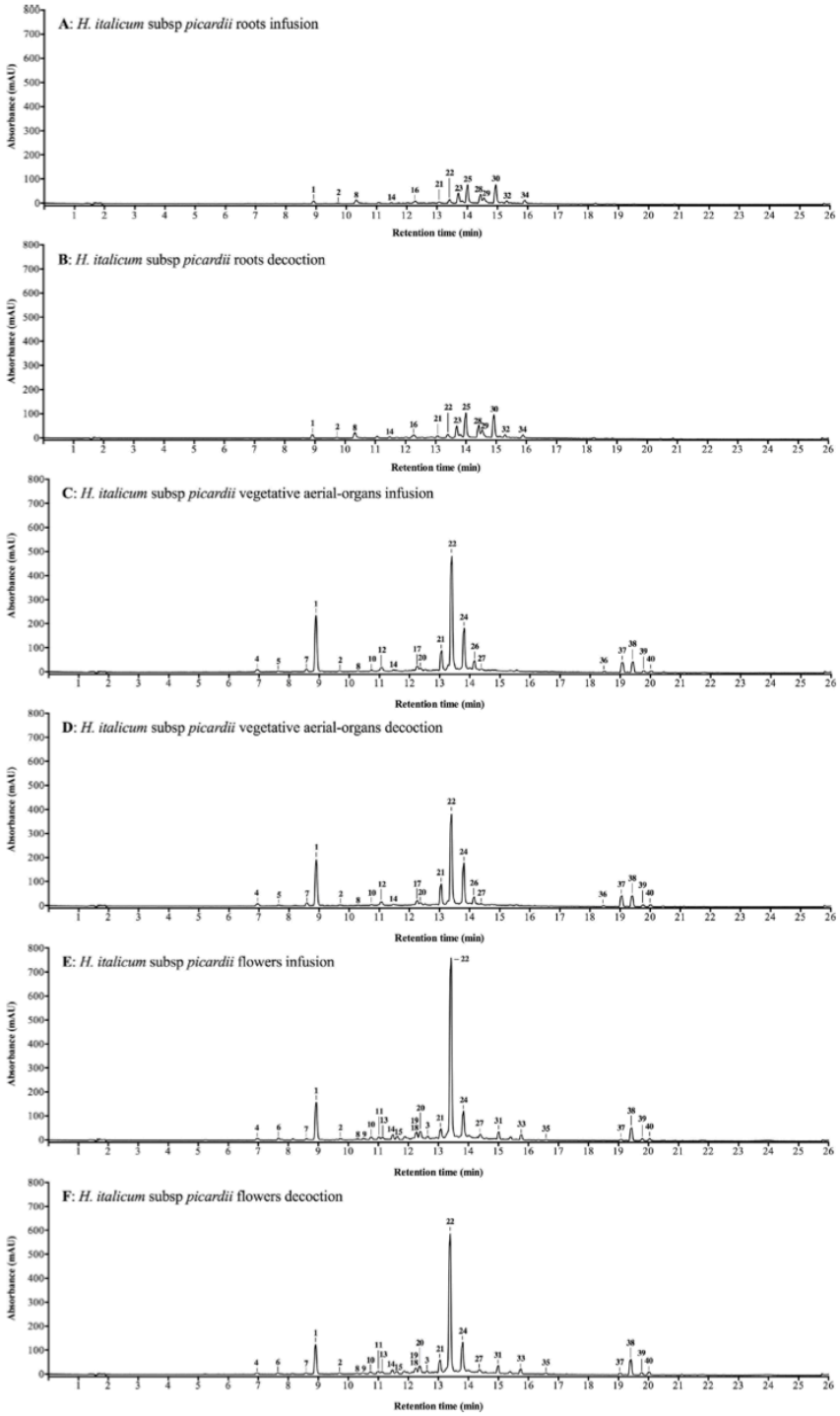

Fig. 1. PDA chromatograms (280 + 330 nm) of the extracts from *H. italicum* subsp. *picardii* roots (A: infusion, B: decoction), vegetative aerial-organs (C: infusion, D: decoction) and flowers (E: infusion, F: decoction). Peak numbers refer to compounds listed in Tables 2 and 3.

Table 3
Relative abundances (%) of the tentatively identified compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs, analysed by LC-PDA-MS. Red = 0%, yellow = 50%, green = 100%; every percentage in between is a mixture of these colours. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

[a]Peak n°	Tentative ID	[b]RT (min)	Roots		Vegetative aerial organs		Flowers		Maximum area detected
			Infusion	Decoction	Infusion	Decoction	Infusion	Decoction	
	Tryptophan	5.26	0.21	0.06	19.17	21.04	100.00	89.98	16 898 458
4	Caffeoylquinic acid isomer	7.01	1.41	2.16	83.32	100.00	62.86	66.15	163 433 756
	*NI ($C_{15}H_{28}O_{10}$)	7.02	NF	NF	20.61	22.33	100.00	92.97	16 451 750
5	Coumaric acid hexoside	7.65	0.27	0.34	88.39	100.00	5.58	6.68	91 587 672
6	Scopoletin hexoside	7.7	1.19	1.19	12.22	13.31	98.87	100.00	193 518 988
	Chlorogenic acid-3-*O*-glucoside	7.78	1.02	0.94	57.56	58.80	100.00	93.73	42 768 967
7	Caffeoylquinic acid isomer	8.63	1.68	3.30	63.77	100.00	27.71	43.43	177 435 679
	Phenylethyl primeveroside	9.67	0.06	0.11	100.00	91.51	8.18	9.13	40 285 455
8	*NI ($C_{24}H_{18}O_{14}$)	10.35	90.71	100.00	24.00	22.14	12.04	14.20	464 514 024
9	*NI ($C_{19}H_{20}O_{11}$)	10.53	4.28	5.62	16.92	19.29	100.00	98.41	47 646 805
10	Feruloylquinic acid	10.79	0.59	0.55	36.73	38.43	98.87	100.00	202 494 981
11	Myricetin glucoside or isomer	11	NF	NF	18.66	26.40	76.79	100.00	92 720 448
12	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	11.1	0.01	0.01	77.20	100.00	4.18	5.81	157 605 397
13	$C_{15}H_{16}O_8$ coupled to 2hexoses, 1deoxyhexose, 1coumaric acid	11.15	NF	NF	0.05	0.37	75.48	100.00	27 088 733
14	*NI ($C_{34}H_{36}O_{19}$)	11.51	1.35	1.66	14.86	20.87	100.00	94.87	173 437 927
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{12}O_5$)	11.6	NF	0.04	86.98	100.00	8.21	10.38	14 262 052
15	*NI ($C_{39}H_{38}O_{23}$)	11.64	NF	NF	NF	0.11	100.00	99.82	66 806 043
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	12.17	0.62	0.63	17.98	27.02	69.54	100.00	25 803 936
16	$C_{14}H_{12}O_4$-*O*-hexoside	12.28	79.73	100.00	14.46	20.39	13.35	17.07	215 945 535
17	Isorhamnetin-*O*-hexoside	12.29	NF	NF	65.02	100.00	3.94	4.94	37 680 334
18	*NI ($C_{24}H_{22}O_{16}$)	12.29	0.19	0.21	12.31	14.10	100.00	89.62	197 543 105
19	*NI ($C_{24}H_{22}O_{17}$)	12.29	0.34	0.20	8.87	11.19	100.00	88.40	75 307 256
20	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	12.44	NF	NF	15.13	24.40	75.96	100.00	246 188 987
21	Dicaffeoylquinic acid	13.1	1.20	1.58	66.49	100.00	36.56	61.17	774 049 004
	Isorhamnetin-*O*-hexoside	13.37	NF	NF	5.42	14.04	83.62	100.00	31 958 060
22	Dicaffeoylquinic acid	13.44	0.95	0.76	64.14	73.25	96.39	100.00	3 585 117 791
23	*NI ($C_{26}H_{30}O_{13}$)	13.74	100.00	97.09	5.64	4.89	1.12	1.10	901 518 717
24	Dicaffeoylquinic acid	13.85	1.17	1.28	71.37	100.00	55.80	78.69	1 636 538 799
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	13.92	0.25	0.29	3.60	5.80	100.00	99.59	49 122 107
25	*NI ($C_{33}H_{22}O_{17}$)	14.02	100.00	64.54	4.02	2.51	0.14	0.25	264 637 977
	Dicaffeoylquinic acid methyl ester	14.07	0.80	1.46	52.17	78.68	63.44	100.00	9 174 467
26	Methoxyoxalyl-dicaffeoylquinic acid	14.17	0.10	0.11	94.00	100.00	2.16	2.10	295 286 636
27	Dicaffeoylquinic acid methyl ester	14.42	0.14	0.17	26.04	26.87	100.00	99.84	101 372 663
28	*NI ($C_{33}H_{22}O_{17}$)	14.45	100.00	71.62	1.63	0.78	NF	NF	165 493 549
29	*NI ($C_{33}H_{22}O_{17}$)	14.57	100.00	66.92	1.88	0.91	NF	NF	144 606 985
	Helichrysin	14.77	NF	NF	80.65	100.00	2.79	4.15	3 404 695
	Dicaffeoylquinic acid methyl ester	14.82	0.90	1.03	59.96	73.11	70.60	100.00	25 279 181
30	*NI ($C_{37}H_{28}O_{19}$)	14.96	100.00	53.47	0.64	0.21	NF	NF	295 393 282
31	Quercetin coupled to coumaric acid and hexose	15.03	NF	NF	0.04	0.07	96.22	100.00	366 986 546
32	*NI ($C_{26}H_{32}O_{13}$)	15.34	100.00	86.95	3.00	3.16	0.26	0.17	178 374 605
33	Tiliroside (kaempferol-3-*O*-*p*-coumaroylglucopyranoside)	15.79	0.01	0.00	0.52	0.90	89.74	100.00	536 636 121
34	*NI ($C_{29}H_{34}O_{16}$)	15.94	100.00	70.98	3.05	2.91	0.29	0.17	334 978 768
	Isomer of naringenin	16.52	NF	NF	89.35	100.00	24.84	31.56	63 885 073
35	Methoxyluteolin	16.58	NF	NF	25.79	35.96	83.25	100.00	29 212 020
	Isomer of naringenin	16.76	0.01	0.01	83.14	100.00	26.41	32.50	178 428 280
	Apigenin	17.79	4.82	3.05	62.44	89.81	73.34	100.00	2 029 743
	Isorhamnetin	17.91	0.89	0.51	66.05	93.53	78.95	100.00	2 525 227
36	Methoxyflavonoid ($C_{16}H_{14}O_5$)	18.5	0.01	0.02	85.11	100.00	7.48	10.98	161 014 058
	4-Hydroxy-3-(3-methyl-2-butenyl) acetophenone	18.66	NF	NF	24.27	20.73	100.00	98.39	5 640 296
37	Pinocembrin	19.08	0.02	0.02	70.77	100.00	16.17	25.25	5 192 433 767
38	Gnaphaliin A	19.43	0.09	0.12	47.20	63.41	60.78	100.00	2 251 428 101
39	Gnaphaliin B	19.81	0.05	0.07	49.77	72.41	56.30	100.00	207 717 204
40	Methoxyflavonoid ($C_{16}H_{12}O_5$)	20.06	0.08	0.10	41.55	64.99	58.76	100.00	1 646 873 985
	Helipyrone	20.92	NF	NF	16.37	8.97	100.00	88.27	39 453 111
	Arzanol	21.66	NF	NF	NF	NF	45.77	100.00	35 054 309
	Methylarzanol	22.03	NF	NF	NF	NF	27.85	100.00	14 237 273

*NI – compound not identified.
NF – not found.
[a]Corresponding peak number in the chromatograms on Fig. 1.
[b]RT – retention times.

Table 4
Antioxidant activity of infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas (% activity in a cup-of-tea): radical scavenging on DPPH, ABTS, NO, $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals, metal chelating activities on copper (CCA) and iron (ICA), and ferric reducing antioxidant power (FRAP). In each column different letters mean significant differences ($p < 0.05$).

Plant/compound			Antioxidant activity (%)							
	Organ	Extract	DPPH	NO	ABTS	$O_2^{\bullet-}$	$OH^{\bullet}$	FRAP	CCA	ICA
H. italicum subsp. *picardii*	Roots	Infusion	74.4 ± 3.56^{d}	62.6 ± 3.26^{bc}	57.6 ± 6.60^{c}	75.1 ± 2.07^{d}	26.0 ± 0.35^{e}	89.2 ± 3.66^{bc}	27.6 ± 2.98^{g}	12.8 ± 2.17^{e}
		Decoction	81.0 ± 0.76^{bc}	64.3 ± 1.91^{b}	66.5 ± 11.1^{b}	87.9 ± 0.81^{b}	19.1 ± 3.53^{f}	76.8 ± 1.45^{d}	37.3 ± 2.70^{f}	17.8 ± 3.24^{de}
	Vegetative aerial-organs	Infusion	82.8 ± 0.38^{ab}	65.3 ± 2.99^{b}	93.1 ± 0.69^{a}	89.7 ± 0.64^{ab}	68.3 ± 1.59^{c}	94.0 ± 6.68^{ab}	63.3 ± 3.46^{de}	25.5 ± 2.21^{c}
		Decoction	83.4 ± 0.65^{ab}	57.8 ± 1.91^{d}	93.1 ± 0.43^{a}	88.8 ± 0.15^{b}	65.8 ± 0.85^{c}	99.7 ± 0.50^{a}	60.8 ± 2.15^{e}	27.5 ± 5.48^{c}
	Flowers	Infusion	85.7 ± 0.29^{a}	63.6 ± 1.89^{b}	93.3 ± 0.38^{a}	91.6 ± 0.13^{a}	77.5 ± 1.18^{b}	82.9 ± 7.20^{cd}	72.2 ± 5.19^{c}	24.1 ± 3.80^{cd}
		Decoction	85.7 ± 0.41^{a}	61.5 ± 1.64^{bcd}	92.7 ± 0.34^{a}	82.6 ± 0.33^{c}	71.2 ± 1.70^{c}	99.8 ± 0.53^{a}	67.8 ± 5.30^{cd}	14.7 ± 3.51^{e}
C. sinensis		Infusion	76.8 ± 3.78^{d}	52.1 ± 0.56^{e}	93.3 ± 0.45^{a}	84.4 ± 1.13^{c}	52.8 ± 1.48^{d}	100 ± 0.00^{a}	80.6 ± 1.73^{b}	41.1 ± 2.87^{b}
		Decoction	77.1 ± 3.79^{cd}	51.5 ± 2.57^{e}	93.1 ± 0.46^{a}	82.1 ± 1.90^{c}	49.1 ± 1.36^{d}	100 ± 0.00^{a}	81.1 ± 0.42^{b}	43.9 ± 4.82^{b}
A. linearis		Infusion	84.6 ± 0.41^{ab}	58.6 ± 0.69^{d}	92.7 ± 0.85^{a}	84.4 ± 0.24^{c}	18.8 ± 2.40^{f}	98.1 ± 2.00^{a}	26.6 ± 3.52^{g}	26.2 ± 1.70^{c}
		Decoction	84.6 ± 0.51^{ab}	59.5 ± 1.29^{cd}	92.9 ± 0.63^{a}	84.9 ± 0.15^{c}	25.6 ± 1.40^{e}	100 ± 0.00^{a}	28.6 ± 3.73^{g}	41.8 ± 4.29^{b}
BHT*			81.7 ± 1.65^{ab}		93.4 ± 0.26^{a}			–		
Ascorbic acid*				90.6 ± 1.35^{a}						
Catechin*						75.2 ± 2.83^{d}	84.4 ± 9.31^{a}			
EDTA*									94.6 ± 0.36^{a}	99.7 ± 0.15^{a}

Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9).
* Positive controls tested at 1 mg/mL (BHT, catechin and EDTA) or 10 mg/mL (ascorbic acid).

and relative abundances. It is out of the scope of the present work to perform a method validation for accurate quantitation of phenolic constituents in *H. italicum* extracts. In Table 2 are the concentrations of phenolics found in the everlasting extracts, using reference standards for quantification. However, in natural products research analytical standards are often very expensive or not commercially available. Therefore, when no standards were available, tentative identification of compounds was accomplished based upon the available chromatographic and spectral information. Orbitrap MS detectors can routinely generate mass spectra with a resolving power up to 140 000 FWHM and obtain mass accuracies within 1–2 ppm; this enables the calculation of the most probable molecular formulae of the generated precursor and product ions [24]. The higher the resolution and the mass accuracy, the more confident compound identification becomes. This utility combined with the selectivity and sensitivity of current hyphenated UHPLC-PDA-MS systems has paved the way towards generic phytochemical analysis [25]. During the current study, 96% and 89% of the mass deviations measured for the precursor ions in HESI negative and HESI positive mode, respectively, were ≤2 ppm. During this study, a hybrid quadrupole-orbital trap MS-analyser (Q Exactive, Thermo Fisher Scientific) was used, which enables selective ion fragmentation. In a selective ion fragmentation experiment, ions of a particular *m*/*z*-range are selected (precursor ions) with a quadrupole and subsequently fragmented into product ions. This functionality contributes significantly to compound identification by generating clean product ion spectra. Selective ion fragmentation is particularly useful for associating product ions with precursor ions during coelution of multiple compounds, as is often the case in complex plant extracts. Data-dependent fragmentation was used to obtain clean product ion spectra of the detected analytes. Product ions are substructures of the precursor ions, formed during fragmentation: structures were assigned to unknown peaks only when both the *m*/*z*-values and molecular formulae/structures of the precursor and product ions were in agreement. Additional information for dereplication was often acquired from PDA spectra, in-house and commercial compound databases (Dictionary of Natural Products [26], ChemSpider [27] and PubChem [28]) and peer reviewed publications. An in-house database with chromatographic and spectral data of reference standards and previously identified compounds was used to compare chromatographic behaviour and product ion spectra of structurally similar compounds found during the current study. These commercial databases allow to find known molecular structures for a most probable molecular formula obtained from a precursor ion. As described by Sumner et al. [29], the metabolomics community consensus is that the leading challenge of metabolomics is the chemically accurate identification of large numbers of metabolites observed in various non-targeted profiling experiments: accurate structure identification requires significant effort which increases dramatically with the increased amount of detected metabolites per analysis. The Chemical Analysis Working Group of the Metabolomics Standards Initiative proposed four identification classes: 1–confident identifications based upon a minimum of two orthogonal data relative to an authentic standard; 2–putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries; 3–putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class); 4–unknown compounds. The compounds identified with reference standards during the present study belong to confidence class 1, while the compounds that were tentatively identified without reference standards belong to classes 2 (reported in *H. italicum* previously) and 3 (not reported in *H. italicum* before). Since different compounds tend to have different ionization efficiencies during LC–MS analysis no absolute quantitative comparison can be made but relative abundances per compound in-between samples can be calculated. Table 3 shows relative abundances of the (tentatively) identified compounds in the everlasting extracts. Diagnostic chromatographic and MS data used for compound identification plus literature used for compound identity confirmation can be found in Table S2 (supplementary material). Fig. 1 represents the extracts' PDA-chromatograms at combined wavelengths (280–330 nm).

A wide versatility of predominantly phenolic constituents was (tentatively) identified in *H. italicum* subsp. *picardii* extracts (Tables 2 and 3). Most phenolics were already described in *Helichrysum* species except for salicylic acid that is, to the best of our knowledge, here firstly described in the genus. For *H. italicum*, no reports were found detailing quinic, protocatechuic, *p*-hydroxybenzoic and syringic acids (but quinic acid derivatives are described in Mari et al. [30]), rutin, apigetrin and quercetin (its glycoside quercetin 3-*O*-glucoside is reported in Mari et al. [30]), which are currently described for the first time in this species. Additionally, Table S2 details the tentatively identified compounds that were already reported in *H. italicum*.

Table 5
Inhibitory activity on microbial α-glucosidase of infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas (% activity in a cup-of-tea). In each column different letters mean significant differences ($p < 0.05$).

Plant/compound	Extract	Organ	Inhibitory activity (%)
H. italicum subsp. *picardii*	Roots	Infusion	22.9 ± 5.6^{g}
		Decoction	31.0 ± 4.7^{f}
	Vegetative aerial-organs	Infusion	41.2 ± 3.5^{e}
		Decoction	45.7 ± 3.9^{de}
	Flowers	Infusion	48.3 ± 5.7^{d}
		Decoction	50.4 ± 3.1^{d}
C. sinensis		Infusion	98.8 ± 0.3^{a}
		Decoction	99.9 ± 0.3^{a}
A. linearis		Infusion	72.3 ± 3.3^{c}
		Decoction	72.7 ± 4.2^{c}
Acarbose*			88.3 ± 0.5^{b}

Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9).
* Positive control at 10 mg/mL.

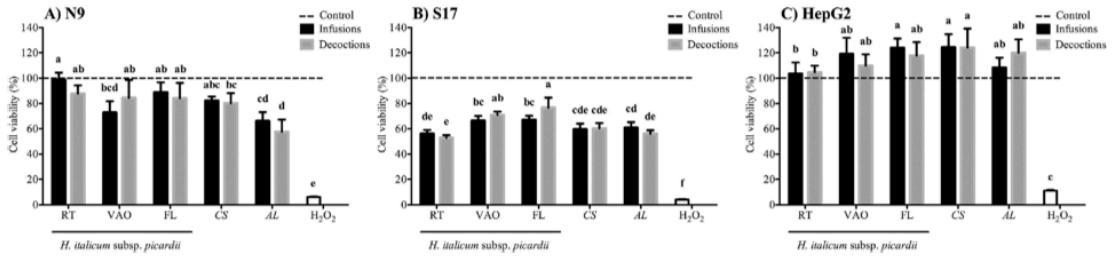

Fig. 2. Toxicity of infusions and decoctions (100 μg/mL extract dw) from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas on mammalian cell lines: A) N9, B) S17 and C) HepG2. Cells treated only with cell culture medium were used as controls; H_2O_2 was used as positive control for cell toxicity. Values represent the mean ± sd of at least three experiments performed in triplicate (n = 9). In each graph different letters mean significant differences ($p < 0.05$). RT: roots, VAO: vegetative aerial-organs, FL: flowers, *CS*: *Camelia sinensis*, *AL*: *Aspalathus linearis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

According to Table 2, a higher diversity of compounds and with consistently higher levels was found in tisanes from vegetative aerial-organs and flowers along with a similarity in the composition of major phenolics in these aboveground organs. The main phenolics detected were quinic and chlorogenic acids, higher in the vegetative aerial-organs (8.2–8.7 and 6.9–7.7 mg/cup-of-tea, respectively) than in the flower extracts (4.7–4.9 and 5.2–6.0 mg/cup-of-tea, respectively). Preferentially detected in flower's tisanes was astragalin (0.49–0.6 mg/cup-of-tea), hyperin and/or isoquercitrin (they are isomers and co-elute; 0.35–0.36 mg/cup-of-tea) and caffeic acid (0.14–0.15 mg/cup-of-tea), while syringic and oleanolic acids were higher in vegetative aerial-organs extracts (0.16–0.17 and 0.11 mg/cup-of-tea, respectively). Table 3 and Fig. 1 also show the composition similarity of major constituents and higher compound diversity of tisanes from vegetative aerial-organs and flowers, with the main tentatively identified compounds being dicaffeoylquinic acid isomers and gnaphaliin A. Note that the "maximum area detected" (Table 3) provides a semi-quantitative information of compound abundance but it should not be interpreted as absolute quantitative comparison since this is not possible based on areas obtained with LC–MS. Composition of root extracts was very different from that of aboveground organs with less abundance and diversity of compounds and, unfortunately, their most abundant compounds (peaks 25 and 30 in Fig. 1) shown in the PDA-chromatograms were not identified (Fig. 1, Table 3). Moreover, some major compounds already reported in *H. italicum* were not detected, such as naringenin-7-*O*-glucoside [31], along with other phenolics compiled in Maksimovic et al. [13], possibly due to the extraction solvent/methods or to the natural phytochemical variations in-between plants, a variability that has been already reported for this species [10,32].

Secondary metabolites like phenolic compounds are implicated in the plant's response to pressures such as predation, infection by pathogens and parasites or wounding, but content in phenolics may also increase under abiotic stress conditions [33]. Extreme temperatures, UV-radiation, salinity or drought are pronounced environmental challenges for halophyte/extremophile plants that live and thrive under such harsh conditions [5,33]. Abiotic stress enhances production and accumulation of reactive oxygen species (ROS) demanding a powerful antioxidant system. As a result, those plants synthesize antioxidant compounds including polyphenolics to counteract ROS and protect cellular structures and metabolic functions from oxidative damage [5,34]. Therefore, the higher phenolic accumulation and assortment in stems & leaves and flower extracts might suggest their protective role was in play possibly against excessive UV-radiation, heat and predation in the aboveground organs.

3.2. Biological activities: in vitro antioxidant and anti-diabetic properties

ROS, such as the superoxide or hydroxyl radicals, are formed naturally in biological systems but an imbalance between the antioxidant defenses and ROS production can result in damaging oxidative stress. This involves damage to cellular macromolecules (like proteins, lipids, DNA) and deregulation of cellular functions with implications in several degenerative and pathological alterations (for example, ageing, cancer, diabetes, and neurodegenerative diseases) [15,35]. However, it is well documented that

antioxidants effectively fight free radicals and oxidative damage and thus they are able to reduce or prevent the severity of different oxidative stress-related diseases [14,35]. Antioxidants are thus an essential group of medicinal preventive molecules and are also used as food additives to prevent harmful modifications of foods which are sensitive to oxidation [5]. In this context, there is a growing economical and security interest on the identification of halophyte species with high antioxidant content aiming at its use in the food industry and in preventive medicine to replace synthetic antioxidants [5].

In this work, the antioxidant potential of a cup-of-tea from *H. italicum* subsp. *picardii* organs was assessed in comparison to those of *C. sinensis* and *A. linearis* and results are summarized on Table 4. Overall, the antioxidant activity of everlasting's vegetative aerial-organs and flowers herbal teas matched or even surpassed that of green teas and rooibos beverages (Table 4). Comparing with *C. sinensis* teas, tisanes from *H. italicum* subsp. *picardii* flowers and stems & leaves were more effective in scavenging DPPH, NO and $OH^{\bullet}$ radicals and had similar RSA against ABTS. In relation to *A. linearis* herbal teas, the same everlasting's tisanes matched its DPPH and ABTS scavenging capacity and surpassed its $OH^{\bullet}$ RSA; from those tisanes, infusions along with roots decoctions were also more active against the NO radical. Moreover, stems & leaves extracts, flowers infusions and roots decoctions had higher $O_2^{\bullet-}$ scavenging activity than both green and herbal red teas. As for the metal-related activities, the capacity to reduce iron (FRAP) was similar between the everlasting's vegetative aerial-organs tisanes and flowers' decoction and the commercial beverages, but green tea was more active in chelating copper and iron. This was probably due to its higher tannin contents since tannins are known metal chelating agents [36]. However, tisanes from *H. italicum* subsp. *picardii* vegetative aerial-organs and flowers were more efficient in chelating copper than rooibos extracts. High antioxidant activity has already been described in *H. italicum* [12,37,38] but studies have seldom focused on aqueous extracts [10] and none was found concerning "cup-of-tea" samples from the different anatomical organs. Our results confirm the strong *in vitro* antioxidant capacity of *H. italicum* subsp. *picardii*, particularly flowers and stems & leaves, and thus show that beverages made from this plant may be useful in preventing oxidative-stress diseases much like the world renowned green tea is reported to be [1,2].

The therapeutic benefits of herbal beverages are related to their high polyphenolic content [4]. Phenolics are recognized powerful antioxidants [5,35] and the everlasting's antioxidant capacity seems to reflect its high phenolic content. In fact, flowers and vegetative aerial-organs were consistently the extracts with higher amounts of TPC, TFC, HAD and flavonols (Table 1) and, except for TPC, they were higher than those detected in the green and herbal red teas. The amount and diversity of phenolics can contribute to the stronger antioxidant activities in tisanes from flowers and stems & leaves: they were determined at higher amounts in these organ's extracts and in greater variety than in roots (Tables 2 and 3). Moreover, the levels of the main components detected in these organs namely quinic and chlorogenic acids, which are reported antioxidant compounds [39,40], were more than 10-fold higher in the above-ground organs. Additionally, some of the other phenolics can have also contributed through addictive and/or synergistic effects. For example, the main tentatively identified compounds dicaffeoylquinic acid isomers are also described as strong antioxidants [41]. Some of the phenolics identified in this everlasting's extracts are described in literature as natural bioactive compounds, which can help explain the plant's medicinal uses. For example, besides the above-mentioned antioxidant compounds, chlorogenic acid has anti-diabetic properties [40], and gnaphaliin, pinocembrin, tiliroside and arzanol have anti-inflammatory activity [10,13]. These and other bioactivities (antiviral, antimicrobial, cytotoxic) have been confirmed in extracts or isolated compounds from other *H. italicum* subspecies [10,13] but, to the best of our knowledge, the present study is the first reporting biological activities and phenolic composition of infusions and decoctions from this everlasting subspecies.

The anti-diabetic potential was assessed through the inhibition of α-glucosidase; the inhibition of such carbohydrate-hydrolyzing enzyme is a therapeutic strategy for the treatment of diabetes mellitus type 2 (T2DM), delaying carbohydrate digestion and reducing postprandial hyperglycemia [31,42]. *H. italicum* subsp. *picardii* tisanes had a moderate to low activity particularly if compared to green tea (Table 5). A "cup-of-tea" from everlasting flowers induced around 50% of α-glucosidase inhibition (infusion: 48.3%, decoction: 50.4%), followed by tisanes from the vegetative aerial-organs (infusion: 41.2%, decoction: 45.7%), while the roots' enzyme inhibition was lowest (infusion: 22.9%, decoction: 31.0%). The current α-glucosidase inhibitory capacity in everlasting's tisanes was lower than reported by Garza et al. [31] in *H. italicum* methanolic extracts but this difference can be ascribed to the different extraction solvents/processes used and/or to a natural variability in secondary metabolites' content. Nevertheless, kaempferol 3-*O*-glucoside (astragalin), presently found at noteworthy concentrations in flowers tisanes (0.49–0.60 mg/cup-of-tea, Table 2), has shown *in vitro* and *in vivo* inhibitory effect on α-glucosidase [43]. Furthermore, chlorogenic acid, one of the main compounds here determined in everlasting's herbal teas, has claimed hypoglycemic and hypolipidemic effects and can regulate glucose and lipid metabolic disorders associated to the progression of diabetes and obesity, among others [40]. Our results thus suggest that *H. italicum* subsp. *picardii* flowers' tisanes can be useful in the control of glucose levels, when used in combined anti-diabetic strategies, by inhibiting dietary carbohydrate digestive enzymes. In fact, Garza et al. [31] also found this anti-diabetic potential in methanolic extracts from *H. italicum*. Moreover, oxidative stress has been found to mediate the effects of diabetes [42] and given the strong antioxidant potential of the everlasting flowers' tisanes, its consumption may also indirectly contribute to prevent or attenuate the disease's symptoms. As expected the commercial teas had a high *in vitro* anti-diabetic potential: *C. sinensis* teas had 99% of α-glucosidase inhibition and *A. linearis* herbal teas 72%. In fact, the consumption of both teas, but especially green tea, is associated with anti-diabetic effects, either as prevention or to ameliorate symptoms associated with T2DM [1,2,23].

3.3. Toxicological evaluation

To ascertain the safety of new products for human consumption, for example plant extracts or herbal beverages, it is crucial to determine their toxicity. Preliminary toxicity screenings of compounds or natural extracts are commonly assessed by *in vitro* methods such as cytotoxicity towards different mammalian cell lines, providing fast and reliable results and reducing *in vivo* testing [6,7,44,45]. In this sense, a preliminary toxicological evaluation of *H. italicum* subsp. *picardii* tisanes was performed on three cell lines to assess cellular viability after incubation with the extracts, alongside with *C. sinensis* and *A. linearis* beverages for comparison. Results are summarized in Fig. 2. The everlasting's extracts had low toxicity with cell viability values similar or higher than those obtained for green and herbal red teas. None of the extracts from the three plants were toxic for hepatocarcinoma (HepG2) cells. For the microglia (N9) cell line, everlasting's tisanes toxicity was very low (>80% viability, except for stems & leaves' infusion which was 73%), as was green tea, while rooibos extracts exerted a moderate toxicity with cell viability between 58% and 66%. Stromal (S17) cells were more sensitive to toxic effects but, nevertheless, everlasting roots' extracts were only moderately toxic (53–56% viability) and not significantly

different from the toxicity exerted by commercial teas (56–61% viability), whereas everlasting's vegetative aerial-organs and flowers had low toxicity (66–77% viability). Overall, these results are quite promising as preliminary toxicological evaluation of the beverages under study, particularly if compared to the ones obtained for the largely consumed green tea and rooibos tisanes, and suggest that these aqueous extracts can be regarded as non-toxic beverages. *In vitro* toxicity studies of *H. italicum* are scarce and include only essential oils and some organic extracts but nevertheless they also indicate a favorable safe profile [10]. However, although *in vitro* cell culture methods are generally accepted as a very effective method for safety testing [45], further experiments on mammalian animal models should be pursued.

4. Conclusion

Our results indicate that infusions and decoctions made from *H. italicum* subsp. *picardii* above-ground organs, particularly flowers, have a high and diverse polyphenolic content, with similar or even higher antioxidant potential than the commercial green and herbal red teas, showing moderate anti-diabetic potential and low toxicity in *in vitro* models. Altogether, our data suggests that everlasting tisanes, especially those from flowers could be further explored as potential health-promoting food additives to be used, for example, in innovative herbal beverages.

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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.jpba.2017.07.007.

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SUPPLEMENTARY MATERIAL

Table S1. Information pertained to the quantification of total polyphenol content (TPC), total flavonoid content (TFC) condensed tannin content (CTC), hydroxycinnamic acid derivatives (HAD) and flavonols: calibration curve equations, linearity, detection limits and limits of quantification.

	Calibration curve equation	Linearity	Detection limits (mg/mL)	Limit of quantification (mg/mL)
TPC	y = 1.2534x + 0.0592	R^2 = 0.9976	0.0	0.0
TFC	y = 0.8952x + 0.0431	R^2 = 0.9990	0.004	0.014
CTC	y = 0.6663x + 0.1081	R^2 = 0.9973	0.004	0.012
HAD	y = 3.5106x + 0.28	R^2 = 0.9986	0.002	0.007
Flavonols	y = 2.5187x + 0.1547	R^2 = 0.9975	0.003	0.010

Table S2. Chromatographic and spectral data of the tentatively identified compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs, detected with a generic LC-PDA-amMS method for moderately polar phytochemicals.
For information regarding the theoretical exact masses, ppm-values of all the precursor ions, and all the most probable molecular formulae calculated for the product ions, please see the Table S2b version.

Compound tentative ID	Most probable molecular formula	HESI neg full MS	HESI neg ddMS^2	HESI pos full MS	HESI pos ddMS^2	[a]RT (min)	Max UV absorb. (nm)	Previously reported
Tryptophan	$C_{11}H_{12}N_2O_2$	203.0819 $[M-H]^-$	188.1; 170.1; 159.1; 146.1; 132.1; 118.1	205.09691 $[M+H]^+$	-	5.26	264; 277; 288	-
Caffeoylquinic acid isomer	$C_{16}H_{18}O_9$	353.08750 $[M-H]^-$	191.1; 179.0; 173.0; 135.0	355.10192 $[M+H]^+$; 377.08400 $[M+Na]^+$	163.0; 145.0; 135.0	7.01	324	[10]
***NI**	$C_{15}H_{28}O_{10}$	367.16071 $[M-H]^-$; 413.16626 $[M-H+FA]^-$	235.1	369.17488 $[M+H]^+$; 386.20143 $[M+NH_4]^+$; 391.15674 $[M+Na]^+$	-	7.02	-	-
Coumaric acid hexoside	$C_{15}H_{18}O_8$	325.09282$[M-H]^-$	163.0; 119.0	344.13343 $[M+NH_4]^+$; 349.08881 $[M+Na]^+$	165.0; 147.0; 119.0	7.65	-	-
Scopoletin hexoside	$C_{16}H_{18}O_9$	399.09296 $[M-H+FA]^-$	191.0; 176.0	355.10168 $[M+H]^+$	193.0; 178.0; 165.0; 137.1; 133.0	7.7	342	-
Chlorogenic acid-3-*O*-glucoside	$C_{22}H_{28}O_{14}$	515.14044 $[M-H]^-$	323.1; 191.1; 179.0; 161.0	517.1551 $[M+H]^+$; 539.13684 $[M+Na]^+$	355.1; 325.1; 163.0	7.78	-	[2]
Caffeoylquinic acid isomer	$C_{16}H_{18}O_9$	353.08750 $[M-H]^-$	191.1; 179.0; 173.0; 135.0	355.10192 $[M+H]^+$; 377.08400 $[M+Na]^+$	163.0; 145.0; 135.0	8.63	327	[10]
Phenylethyl primeveroside	$C_{19}H_{28}O_{10}$	415.161 $[M-H]^-$; 461.16663 $[M-H+FA]^-$	-	434.2017 $[M+NH_4]^+$; 439.15684 $[M+Na]^+$	295.1; 259.1; 145.0; 133.0	9.67	-	-
***NI**	$C_{24}H_{18}O_{14}$	529.06263 $[M-H]^-$	369.0; 351.0; 191.0; 179.0; 177.0; 161.0; 147.0; 133.0	531.07652 $[M+H]^+$; 548.10317 $[M+NH_4]^+$	163.0	10.35	249; 305; 397	-
***NI**	$C_{19}H_{20}O_{11}$	423.09337 $[M-H]^-$	261.0; 217.0; 203.0; 187.0; 173.1; 159.0; 135.0; 109.0	425.10725 $[M+H]^+$; 447.08920 $[M+Na]^+$	263.0; 217.0; 199.0; 189.0; 173.1; 171.0; 163.0; 161.1; 143.0; 115.0	10.53	372	-
Feruloylquinic acid	$C_{17}H_{20}O_9$	367.10366 $[M-H]^-$	191.1; 173.0; 134.0; 111.0	369.11763 $[M+H]^+$; 391.09969 $[M+Na]^+$	177.0; 145.0; 117.0	10.79	298; 326	-
Myricetin glucoside or isomer	$C_{21}H_{20}O_{13}$	479.08311 $[M-H]^-$; 525.08908 $[M-H+FA]^-$	317.0	481.0973 $[M+H]^+$	319.0	11	-	[2][10]
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 $[M-H]^-$	301.0	465.10247 $[M+H]^+$	303.0	11.1	342	[3]
$C_{15}H_{10}O_8$ coupled to 2hexoses, 1deoxyhexose, 1coumaric acid	$C_{42}H_{46}O_{24}$	933.23289 $[M-H]^-$; 787.17444 [M-H-deoxyhexose]$^-$	625.1; 479.1; 317.0; 287.0; 271.0; 166.0	935.24414 $[M+H]^+$; 789.18698 [M+H-deoxyhexose]$^+$	481.1; 319.0; 147.0	11.15	286; 310	-
***NI**	$C_{34}H_{36}O_{19}$	747.17914 $[M-H]^-$	585.1	749.19212 $[M+H]^+$	587.1; 425.1; 325.1; 307.1; 263.0; 217.0; 199.0; 163.0	11.51	339	-
Flavonoid-O-hexoside (flavonoid aglycon: $C_{15}H_{12}O_5$)	$C_{21}H_{22}O_{10}$	433.11402 $[M-H]^-$; 479.11988 $[M-H+FA]^-$	271.1	435.12808 $[M+H]^+$; 457.24027 $[M+Na]^+$	273.1; 255.1; 227.1; 199.1; 153.0; 149.0	11.6	-	-
***NI**	$C_{39}H_{38}O_{23}$	873.17321 $[M-H]^-$	829.2; 625.1; 479.1; 317.0; 287.0; 271.0; 166.0	875.18622 $[M+H]^+$	567.01; 319.0; 147.0	11.64	283; 318	-
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 $[M-H]^-$	301.0; 175.0; 151.0	465.10247 $[M+H]^+$	303.0	12.17	-	[3]

$C_{14}H_{12}O_4$-*O*-hexoside	$C_{20}H_{22}O_9$	451.1245 $[M-H+FA]^-$	243.1; 199.1; 167.0; 143.0; 119.0	407.13317 $[M+H]^+$; 429.11503 $[M+Na]^+$	245.1; 217.1; 147.0	12.28	354	-
Isorhamnetin-*O*-hexoside	$C_{16}H_{12}O_7$	477.10383 $[M-H]^-$	462.1; 315.0; 299.0; 271.0	479.11782 $[M+H]^+$	317.1; 302.0	12.29	-	[3]
***NI**	$C_{24}H_{22}O_{16}$	565.0834 $[M-H]^-$	521.1; 317.0; 166.0	567.09721 $[M+H]^+$	-	12.29	-	-
***NI**	$C_{24}H_{22}O_{17}$	581.07844 $[M-H]^-$	519.1; 315.0; 287.0; 255.0	583.0922 $[M+H]^+$	335.0; 317.0; 289.0	12.29	-	-
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 $[M-H]^-$	301.0	465.10247 $[M+H]^+$	303.0	12.44	341	[3]
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	13.1	300; 328	[3][10]
Isorhamnetin-*O*-hexoside	$C_{16}H_{12}O_7$	477.1034 $[M-H]^-$	314.0; 299.0; 285.0; 271.0; 257.0; 243.0	479.11767 $[M+H]^+$	317.1; 302.0; 285.0; 274.0; 257.0	13.37	-	[3]
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 161.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	13.44	300; 328	[3][10]
***NI**	$C_{26}H_{30}O_{13}$	549.16129 $[M-H]^-$	243.1; 228.0; 199.1; 167.0; 143.0; 119.0	551.17521 $[M+H]^+$; 573.15673 $[M+Na]^+$	245.1; 217.1; 147.0	13.74	351	-
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 $[M-H]^-$	353.1; 191.1; 179.0; 173.0; 161.0; 155.0; 135.0	517.13405 $[M+H]^+$	163.0; 145.0; 135.0; 117.0	13.85	300; 328	[3][10]
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 $[M-H]^-$	301.0; 179.0; 151.0	465.10247 $[M+H]^+$	303.0	13.92	-	[3]
***NI**	$C_{33}H_{22}O_{17}$	689.07898 $[M-H]^-$	529.1; 351.0; 245.0; 217.0; 201.1; 189.1; 179.0; 177.0; 173.1; 161.0; 135.0; 133.0; 105.0	691.09231 $[M+H]^+$	513.1; 307.0; 179.0; 163.0; 161.0; 149.0; 145.0; 135.0; 133.0; 123.0; 117.0; 105.0	14.02	251; 327; 391	-
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 191.1; 179.0; 173.0; 161.0; 135.0	531.14914 $[M+H]^+$	-	14.07	-	[3]
Methoxyoxalyl-dicaffeoylquinic acid	$C_{28}H_{26}O_{15}$	601.11993 $[M-H]^-$	557.1; 395.1; 233.1; 191.1; 179.0; 173.0	603.13365 $[M+H]^+$; 620.16000 $[M+NH4]^+$; 625.11521 $[M+Na]^+$	423.1; 163.0; 145.0; 135.0	14.17	328	-
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 353.1; 191.1; 179.0; 161.0; 134.0	531.14914 $[M+H]^+$	-	14.42	-	[3]
***NI**	$C_{33}H_{22}O_{17}$	689.07898 $[M-H]^-$	529.1; 351.0; 245.0; 217.0; 201.1; 189.1; 179.0; 177.0; 173.1; 161.0; 135.0; 133.0; 105.0	691.09231 $[M+H]^+$	321.0; 295.01; 249.1; 181.0; 163.0; 151.0; 145.0; 135.0; 133.0; 123.0; 117.0; 105.0	14.45	251; 323; 389	-
***NI**	$C_{33}H_{22}O_{17}$	689.07898 $[M-H]^-$	529.1; 511.0; 351.0; 245.0; 217.0; 201.1; 189.1; 179.0; 177.0; 173.1; 161.0; 135.0; 133.0; 105.0	691.09231 $[M+H]^+$	295.1; 249.1; 181.0; 163.0; 151.0; 145.0; 135.0; 133.0; 123.0; 117.0; 105.0	14.57	252; 306; 327; 395	-
Helichrysin	$C_{22}H_{24}O_{10}$	447.12967 $[M-H]^-$	285.1; 269.0; 241.0; 179.0	449.14537 $[M+H]^+$	-	14.77	-	[10]
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 $[M-H]^-$	367.1; 353.1; 191.1; 179.0; 173.0; 161.0; 135.0	531.14914 $[M+H]^+$	-	14.82	-	[3]
***NI**	$C_{37}H_{28}O_{19}$	775.1161 $[M-H]^-$	615.1; 437.1; 375.1; 331.1; 245.0; 227.0; 217.0; 201.1; 199.0; 189.1; 179.0; 177.0; 173.1; 161.0; 135.0; 133.0; 105.0	777.12893 $[M+H]^+$; 794.15571 $[M+Na]^+$	583.1; 321.0; 295.1; 293.0; 277.0; 267.1; 265.0; 251.1; 249.1; 233.1; 221.1; 179.0; 163.0; 151.0; 149.0; 145.0; 135.0; 133.0; 123.0; 121.0; 117.0; 105.0	14.96	250; 307; 328; 400	-
Quercetin coupled to coumaric acid and hexose	$C_{30}H_{26}O_{14}$	609.12506 $[M-H]^-$	463.1; 300.0; 271.0; 255.0; 179.1; 151.0	611.13859 $[M+H]^+$; 633.12037 $[M+Na]^+$	303.0	15.03	271; 308	-
***NI**	$C_{26}H_{32}O_{13}$	551.17728 $[M-H]^-$	213.1; 183.1; 175.0; 173.0; 159.0; 141.1; 113.0	575.17289 $[M+Na]^+$	-	15.34	259; 270; 291; 307; 326; 349	
Tiliroside (kaempferol-3-*O*-*p*-coumaroylglucopyranoside)	$C_{30}H_{26}O_{13}$	593.13006 $[M-H]^-$	447.1; 285.0; 255.0; 227.0; 145.0	595.14372 $[M+H]^+$; 617.12527 $[M+Na]^+$	309.1; 287.0; 147.0; 119.0	15.79	314	[3][7]

***NI**	$C_{29}H_{34}O_{16}$	637.17784 $[M-H]^-$	593.2; 235.0; 213.1; 195.1; 183.1; 175.0; 173.0; 159.0; 141.1; 113.0	639.19099 $[M+H]^+$; 656.21758 $[M+NH_4]^+$; 661.17295 $[M+Na]^+$	425.1; 283.1; 245.1; 197.1; 178.1; 169.1; 152.1; 141.1; 115.0; 101.0	15.94	259; 270; 290; 307; 327; 349	
Isomer of naringenin	$C_{15}H_{12}O_5$	271.06111 $[M-H]^-$	253.0; 225.0; 197.1; 161.1; 151.0; 125.0; 107.0	273.07509 $[M+H]^+$	255.1; 227.1; 199.1; 153.0; 149.0	16.52	291	-
Methoxyluteolin	$C_{16}H_{12}O_7$	315.05113 $[M-H]^-$	300.0; 287.1; 181.0; 166.1; 153.0	317.06498 $[M+H]^+$	302.0	16.58	-	[2]
Isomer of naringenin	$C_{15}H_{12}O_5$	271.06111 $[M-H]^-$	253.0; 225.1; 197.1; 161.1; 151.0; 125.0; 107.0	273.07509 $[M+H]^+$	255.1; 227.1; 199.1; 153.0; 149.0	16.76	291	-
Apigenin	$C_{15}H_{10}O_5$	269.04555 $[M-H]^-$	-	271.06010 $[M+H]^+$	-	17.79	-	[9]
Isorhamnetin	$C_{16}H_{12}O_7$	315.05103 $[M-H]^-$	-	317.06558 $[M+H]^+$	-	17.91	-	-
Methoxyflavonoid ($C_{16}H_{14}O_5$)	$C_{16}H_{14}O_5$	285.07683 $[M-H]^-$	270.0	287.0907 $[M+H]^+$	271.1	18.5	291; 347	-
4-Hydroxy-3-(3-methyl-2-butenyl) acetophenone	$C_{13}H_{16}O_2$	203.10775 $[M-H]^-$	148.0	205.12197 $[M+H]^+$	149.1	18.66	-	[6]
Pinocembrin	$C_{15}H_{12}O_4$	255.06628 $[M-H]^-$	213.1; 185.1; 171.0 107.0	257.08021 $[M+H]^+$	215.1; 173.1; 153.0; 131.0; 103.0	19.08	289	[7]
Gnaphaliin A	$C_{17}H_{14}O_6$	313.07176 $[M-H]^-$	298.0; 283.0; 255.0; 227.0	315.08631 $[M+H]^+$	300.1; 285.0; 257.0	19.43	273; 353	[4][6]
Gnaphaliin B	$C_{17}H_{14}O_6$	313.07176 $[M-H]^-$	298.0; 283.02; 255.0; 227.0	315.08631 $[M+H]^+$	299.0; 285.0; 282.0; 257.0; 254.1	19.81	270; 318	[4][6]
Methoxyflavonoid ($C_{16}H_{12}O_5$)	$C_{16}H_{12}O_5$	283.06089 $[M-H]^-$; 567.12932 $[2M-H]^-$	268.0; 239.0; 211.0	285.07505 $[M+H]^+$	270.0	20.06	-	-
Helipyrone	$C_{17}H_{20}O_6$	319.11839 $[M-H]^-$	153.1; 109.1	321.1329 $[M+H]^+$	167.1; 155.1	20.92	-	[5][8]
Arzanol	$C_{22}H_{26}O_7$	401.16023 $[M-H]^-$	235.1; 179.0; 153.1; 109.1	403.1744 $[M+H]^+$	-	21.66	-	[1][5][8]
Methylarzanol	$C_{23}H_{28}O_7$	415.17611 $[M-H]^-$	249.1; 193.05; 153.1; 109.1	417.19024 $[M+H]^+$	-	22.03	-	[5]

*NI – compound not identified

[a] RT – retention times

Table S2b. Chromatographic and spectral data of the tentatively identified compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs, detected with a generic LC-PDA-amMS method for moderately polar phytochemicals. Complete version with information regarding the theoretical exact masses, ppm-values of all the precursor ions, and all the most probable molecular formulae calculated for the product ions.

		HESI neg		
Compound tentative ID	Most probable molecular formula	full MS	mass deviation (in ppm)	ddMS^2
Tryptophan	$C_{11}H_{12}N_2O_2$	203.0819 [M-H]$^-$	-3.4	188.07033 (C11 H10 O2 N); 170.06004 (C11 H8 O N); 159.09167 (C10 H11 N2); 146.06004 (C9 H8 O N); 132.08078 (C9 H10 N); 118.06513 (C8 H8 N)
Caffeoylquinic acid isomer	$C_{16}H_{18}O_9$	353.08750 [M-H]$^-$	-0.9	191.05608 (C7 H11 O6); 179.03418 (C9 H7 O4); 173.04472 (C7 H9 O5); 135.04442 (C8 H7 O2)
*NI	$C_{15}H_{23}O_{10}$	367.16071 [M-H]$^-$; 413.16626 [M-H+FA]$^-$	-0.7	235.11852 (C10 H19 O6)
Coumaric acid hexoside	$C_{15}H_{18}O_8$	325.09282[M-H]$^-$	-0.2	163.03926 (C9 H7 O3); 119.04895 (C8 H7 O)
Scopoletin hexoside	$C_{16}H_{18}O_9$	399.09296 [M-H+FA]$^-$	-0.8	191.03452 (C10 H7 O4); 176.01106 (C9 H4 O4)
Chlorogenic acid-3-*O*-glucoside	$C_{22}H_{28}O_{14}$	515.14044 [M-H]$^-$	-0.4	323.07748 (C15 H15 O8); 191.05559 (C7 H11 O6); 179.03479 (C9 H7 O4); 161.02357 (C9 H5 O3)
Caffeoylquinic acid isomer	$C_{16}H_{18}O_9$	353.08750 [M-H]$^-$	-0.9	191.05608 (C7 H11 O6); 179.03418 (C9 H7 O4); 173.04472 (C7 H9 O5); 135.04442 (C8 H7 O2)
Phenylethyl primeveroside	$C_{19}H_{28}O_{10}$	415.161 [M-H]$^-$; 461.16663 [M-H+FA]$^-$	0.1	-
*NI	$C_{28}H_{34}O_{14}$	529.06263 [M-H]$^-$	0.5	369.04599 (C15 H13 O11); 351.03629 (C15 H11 O10); 191.01894 (C6 H7 O7); 179.03413 (C9 H7 O4); 177.01860 (C9 H5 O4); 161.02350 (C9 H5 O3); 147.02840 (C5 H7 O5); 133.02856 (C8 H5 O2)
*NI	$C_{19}H_{20}O_{11}$	423.09337 [M-H]$^-$	0.2	261.03958 (C13 H9 O6); 217.05038 (C12 H9 O4); 203.03444 (C11 H7 O4); 187.03941 (C11 H7 O3); 173.06029 (C11 H9 O2); 159.04428 (C10 H7 O2); 135.04442 (C8 H7 O2); 109.02841 (C6 H5 O2)
Feruloylquinic acid	$C_{17}H_{20}O_9$	367.10366 [M-H]$^-$	0.5	191.05542 (C7 H11 O6); 173.04478 (C7 H9 O5); 134.03633 (C8 H6 O2); 111.04389 (C6 H7 O2)
Myricetin glucoside or isomer	$C_{21}H_{20}O_{13}$	479.08311 [M-H]$^-$; 525.08908 [M-H+FA]$^-$	0.0	317.03021
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 [M-H]$^-$	0.1	301.03503 (C15 H9 O7)
$C_{15}H_{10}O_8$ coupled to 2hexoses, 1deoxyhexose, 1coumaric acid	$C_{42}H_{46}O_{24}$	933.23289 [M-H]$^-$; 787.17444 [M-H-deoxyhexose	2.4	625.12079 (C30 H25 O15); 479.08301 (C21 H19 O13); 317.02972 (C15 H9 O8); 287.02002 (C14 H7 O7); 271.02530 (C14 H7 O6); 165.98988 (C7 H2 O5)
*NI	$C_{34}H_{36}O_{19}$	747.17914 [M-H]$^-$	1.8	585.14258 (C32 H25 O11);
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{12}O_6$)	$C_{21}H_{22}O_{11}$	433.11402 [M-H]$^-$; 479.11988 [M-H+FA]$^-$	0.0	271.06161 (C15 H11 O5)
*NI	$C_{39}H_{38}O_{23}$	873.17321 [M-H]$^-$	0.1	829.18298 (C38 H37 O21); 625.12079 (C30 H25 O15); 479.08301 (C21 H19 O13); 317.02972 (C15 H9 O8); 287.02002 (C14 H7 O7); 271.02530 (C14 H7 O6); 165.98988 (C7 H2 O5)
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 [M-H]$^-$	0.1	301.03525 (C15 H9 O7); 175.00365 (C9 H3 O4); 151.00252 (C7 H3 O4)
$C_{14}H_{12}O_8$-*O*-hexoside	$C_{20}H_{22}O_{13}$	451.1245 [M-H+FA]$^-$	-0.2	243.06618 (C14 H11 O4); 199.07590 (C13 H11 O2); 167.04871 (C12 H7 O); 143.04927 (C10 H7 O); 119.04881 (C8 H7 O)
Isorhamnetin-*O*-hexoside	$C_{22}H_{22}O_{12}$	477.10383 [M-H]$^-$	0.0	462.08203 (C21 H18 O12); 315.05075 (C16 H11 O7); 299.01974 (C15 H7 O7); 271.02515 (C14 H7 O6);
*NI	$C_{28}H_{22}O_{16}$	565.0834 [M-H]$^-$	-0.2	521.09387 (C23 H21 O14); 317.02994 (C15 H9 O8); 165.98996 (C7 H2 O5)
*NI	$C_{28}H_{22}O_{17}$	581.07844 [M-H]$^-$	0.0	519.07874 (C23 H19 O14); 315.01495 (C15 H7 O8); 287.01974 (C14 H7 O7); 255.02971 (C14 H7 O5)
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 [M-H]$^-$	0.1	301.03552 (C15 H9 O7)
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 [M-H]$^-$	0.0	353.08781 (C16 H17 O9); 191.05611 (C7 H11 O6); 179.03498 (C9 H7 O4); 173.04486 (C7 H9 O5); 161.02354 (C9 H5 O3); 155.03398 (C7 H7 O4); 135.04515 (C8 H7 O2)
Isorhamnetin-*O*-hexoside	$C_{22}H_{22}O_{12}$	477.1034 [M-H]$^-$	-0.9	314.04358 (C16 H10 O7); 299.02063 (C15 H7 O7); 285.04095 (C15 H9 O6); 271.02499 (C14 H7 O6); 257.04584 (C14 H9 O5); 243.02968 (C13 H7 O5)
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 [M-H]$^-$	0.0	353.08781 (C16 H17 O9); 191.05611 (C7 H11 O6); 179.03498 (C9 H7 O4); 161.02354 (C9 H5 O3); 135.04515 (C8 H7 O2)
*NI	$C_{30}H_{30}O_{13}$	549.16129 [M-H]$^-$	-0.1	243.06618 (C14 H11 O4); 228.04265 (C13 H8 O4); 199.07590 (C13 H11 O2); 167.04871 (C12 H7 O); 143.04927 (C10 H7 O); 119.04881 (C8 H7 O)
Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	515.11950 [M-H]$^-$	0.0	353.08781 (C16 H17 O9); 191.05611 (C7 H11 O6); 179.03498 (C9 H7 O4); 173.04486 (C7 H9 O5); 161.02354 (C9 H5 O3); 155.03398 (C7 H7 O4); 135.04515 (C8 H7 O2)
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	$C_{21}H_{20}O_{12}$	463.08824 [M-H]$^-$	0.1	301.03531 (C15 H9 O7); 178.99783 (C8 H3 O5); 151.00256 (C7 H3 O4)
*NI	$C_{33}H_{32}O_{17}$	689.07898 [M-H]$^-$	0.8	1.06366 (C24 H17 O14); 351.03662 (C15 H11 O10); 245.04507 (C13 H9 O5); 217.05005 (C12 H9 O4); 201.05527 (C12 H9 O3); 189.05510 (C11 H9 O3); 179.03430 (C9 H7 O4); 177.01865 (C9 H5 O4); 173.06023 (C11 H9 O2); 161.02362 (C9 H5 O3); 135.04422 (C8 H7 O2); 133.02853 (C8 H5 O2); 105.03326 (C7 H5
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 [M-H]$^-$	0.0	367.10382 (C17 H19 O9); 191.05547 (C7 H11 O6); 179.03398 (C9 H7 O4); 173.04486 (C7 H9 O5); 161.02332 (C9 H5 O3); 135.04430 (C8 H7 O2)
Methoxyoxalyl-dicaffeoylquinic acid	$C_{28}H_{26}O_{15}$	601.11993 [M-H]$^-$	0.1	557.12866 (C27 H25 O13); 395.09753 (C18 H19 O10); 233.06639 (C9 H13 O7); 191.05615 (C7 H11 O6); 179.03410 (C9 H7 O4); 173.04485 (C7 H9 O5)
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 [M-H]$^-$	0.0	367.10382 (C17 H19 O9); 353.08881 (C16 H17 O9); 191.05547 (C7 H11 O6); 179.03398 (C9 H7 O4); 161.02332 (C9 H5 O3); 134.03641 (C8 H6 O2)
*NI	$C_{33}H_{32}O_{17}$	689.07898 [M-H]$^-$	0.8	1.06366 (C24 H17 O14); 351.03662 (C15 H11 O10); 245.04507 (C13 H9 O5); 217.05005 (C12 H9 O4); 201.05527 (C12 H9 O3); 189.05510 (C11 H9 O3); 179.03430 (C9 H7 O4); 177.01865 (C9 H5 O4); 173.06023 (C11 H9 O2); 161.02362 (C9 H5 O3); 135.04422 (C8 H7 O2); 133.02853 (C8 H5 O2); 105.03326 (C7 H5
*NI	$C_{33}H_{32}O_{17}$	689.07898 [M-H]$^-$	0.8	1.17 O14); 511.05008 (C24 H15 O13); 351.03662 (C15 H11 O10); 245.04507 (C13 H9 O5); 217.05005 (C12 H9 O4); 201.05527 (C12 H9 O3); 189.05510 (C11 H9 O3); 179.03430 (C9 H7 O4); 177.01865 (C9 H5 O4); 173.06023 (C11 H9 O2); 161.02362 (C9 H5 O3); 135.04422 (C8 H7 O2); 133.02853 (C8 H5 O2); 105
Helichrysin	$C_{22}H_{24}O_{10}$	447.12967 [M-H]$^-$	0.0	285.07614 (C16 H13 O5); 269.04584 (C15 H9 O5); 241.05122 (C14 H9 O4); 179.03452 (C9 H7 O4)
Dicaffeoylquinic acid methyl ester	$C_{26}H_{26}O_{12}$	529.13515 [M-H]$^-$	0.0	367.10382 (C17 H19 O9); 353.08881 (C16 H17 O9); 191.05547 (C7 H11 O6); 179.03398 (C9 H7 O4); 173.04486 (C7 H9 O5); 161.02332 (C9 H5 O3); 135.04430 (C8 H7 O2)
*NI	$C_{37}H_{38}O_{19}$	775.1161 [M-H]$^-$	1.2	375.07431 (C18 H15 O9); 331.08240 (C17 H15 O7); 245.04507 (C13 H9 O5); 227.03461 (C13 H7 O4); 217.05005 (C12 H9 O4); 201.05527 (C12 H9 O3); 199.03969 (C12 H7 O3); 189.05510 (C11 H9 O3); 179.03430 (C9 H7 O4); 177.01865 (C9 H5 O4); 173.06023 (C11 H9 O2); 161.02362 (C9 H5 O3); 135.04422 (C8
Quercetin coupled to coumaric acid and hexose	$C_{30}H_{26}O_{14}$	609.12506 [M-H]$^-$	0.1	463.09033 (C21 H19 O12); 300.02765 (C15 H8 O7); 271.02478 (C14 H7 O6); 255.03011 (C14 H7 O5); 178.99759 (C8 H3 O5); 151.00258 (C7 H3 O4)
*NI	$C_{26}H_{28}O_{13}$	551.17728 [M-H]$^-$	0.5	213.09189 (C14 H13 O2); 183.08060 (C13 H11 O); 175.02460 (C6 H7 O6); 173.04486 (C7 H9 O5); 159.02885 (C6 H7 O5); 141.06989 (C11 H9); 113.02332 (C5 H5 O3)
Tiliroside (kaempferol-3-*O*-*p*-coumaroylglucopyranoside)	$C_{30}H_{26}O_{13}$	593.13006 [M-H]$^-$	0.0	447.09265 (C21 H19 O11); 285.04004 (C15 H9 O6); 255.02991 (C14 H7 O5); 227.03481 (C13 H7 O4); 145.02840 (C9 H5 O2)
*NI	$C_{29}H_{30}O_{16}$	637.17784 [M-H]$^-$	0.7	593.18823 (C28 H33 O14); 235.04659 (C8 H11 O8); 213.09189 (C14 H13 O2); 195.08133 (C14 H11 O); 183.08060 (C13 H11 O); 175.02460 (C6 H7 O6); 173.04486 (C7 H9 O5); 159.02885 (C6 H7 O5); 141.06989 (C11 H9); 113.02332 (C5 H5 O3)
Isomer of naringenin	$C_{15}H_{12}O_5$	271.06111 [M-H]$^-$	-0.3	253.05063 (C15 H9 O4); 225.05530 (C14 H9 O3); 197.06038 (C13 H9 O2); 161.06000 (C10 H9 O2); 151.00255 (C7 H3 O4); 125.02344 (C6 H5 O3); 107.01259 (C6 H3 O2)
Methoxyluteolin	$C_{16}H_{12}O_7$	315.05113 [M-H]$^-$	0.3	300.02753 (C15 H8 O7); 287.05630 (C15 H11 O6); 181.01355 (C8 H5 O5); 165.99005 (C7 H2 O5); 153.01807 (C7 H5 O4)
Isomer of naringenin	$C_{15}H_{12}O_5$	271.06111 [M-H]$^-$	-0.3	253.05063 (C15 H9 O4); 225.05530 (C14 H9 O3); 197.06038 (C13 H9 O2); 161.06000 (C10 H9 O2); 151.00255 (C7 H3 O4); 125.02344 (C6 H5 O3); 107.01259 (C6 H3 O2)
Apigenin	$C_{15}H_{10}O_5$	269.04555 [M-H]$^-$	0.0	-
Isorhamnetin	$C_{16}H_{12}O_7$	315.05103 [M-H]$^-$	0.0	-
Methoxyflavonoid ($C_{16}H_{14}O_6$)	$C_{16}H_{14}O_6$	285.07683 [M-H]$^-$	-0.1	270.05368 (C15 H10 O5)
4-Hydroxy-3-(3-methyl-2-butenyl) acetophenone	$C_{13}H_{16}O_2$	203.10775 [M-H]$^-$	0.0	148.0517 (C9 H8 O2)
Pinocembrin	$C_{15}H_{12}O_4$	255.06628 [M-H]$^-$	0.0	213.05603 (C13 H9 O3); 185.05997 (C12 H9 O2); 171.04442 (C11 H7 O2); 107.01263 (C6 H3 O2)
Gnaphaliin A	$C_{17}H_{14}O_6$	313.07176 [M-H]$^-$	0.0	298.04831 (C16 H10 O6); 283.02499 (C15 H7 O6); 255.02983 (C14 H7 O5); 227.03473 (C13 H7 O4)
Gnaphaliin B	$C_{17}H_{14}O_6$	313.07176 [M-H]$^-$	0.0	298.04831 (C16 H10 O6); 283.02499 (C15 H7 O6); 255.02983 (C14 H7 O5); 227.03473 (C13 H7 O4)
Methoxyflavonoid ($C_{16}H_{12}O_5$)	$C_{16}H_{12}O_5$	283.06089 [M-H]$^-$; 567.12932 [2M-H]$^-$	-1.1	268.03796 (C15 H8 O5); 239.03476 (C14 H7 O4); 211.03970 (C13 H7 O3)
Helipyrone	$C_{17}H_{20}O_6$	319.11839 [M-H]$^-$	-1.0	153.05466 (C8 H9 O3); 109.06475 (C7 H9 O)
Arzanol	$C_{22}H_{30}O_7$	401.16023 [M-H]$^-$	-0.9	235.09735 (C13 H15 O4); 179.03410 (C9 H7 O4); 153.05464 (C8 H9 O3); 109.06473 (C7 H9 O)
Methylarzanol	$C_{23}H_{30}O_7$	415.17611 [M-H]$^-$	-0.3	249.11311 (C14 H17 O4); 193.05000 (C10 H9 O4); 153.05470 (C8 H9 O3); 109.06479 (C7 H9 O)

Table S2b. (cont.)

Compound tentative ID	HESI pos			RT (min)	Max UV absorbance (nm)	Previously reported
	full MS	mass deviation (in	ddMS^2			
Tryptophan	205.0969 [M+H]$^+$	-1.2	-	5.26	264; 277; 288	-
Caffeoylquinic acid isomer	355.10192 [M+H]$^+$; 377.08400 [M+Na]$^+$	-1.2	163.03897 (C9 H7 O3); 145.02823 (C9 H5 O2); 135.04395 (C8 H7 O2)	7.01	324	[10]
*NI	369.17488 [M+H]$^+$; 386.20143 [M+NH$_4$]$^+$; 391.15674 [M+Na]$^+$	-1.7	-	7.02	-	-
Coumaric acid hexoside	344.13343 [M+NH$_4$]$^+$; 349.08881 [M+Na]$^+$	-1.6	165.05443 (C9 H9 O3); 147.04387 (C9 H7 O2); 119.04935 (C8 H7 O)	7.65	-	-
Scopoletin hexoside	355.10168 [M+H]$^+$	-1.9	193.04929 (C10 H9 O4); 178.02576 (C9 H6 O4); 165.05441 (C9 H9 O3); 137.05959 (C8 H9 O2); 133.02835 (C8 H5 O2)	7.7	342	-
Chlorogenic acid-3-*O*-glucoside	517.1551 [M+H]$^+$; 539.13684 [M+Na]$^+$	-0.2	355.10107 (C16 H19 O9); 325.09076 (C15 H17 O8); 163.03870	7.78	-	[2]
Caffeoylquinic acid isomer	355.10192 [M+H]$^+$; 377.08400 [M+Na]$^+$	-1.2	163.03897 (C9 H7 O3); 145.02823 (C9 H5 O2); 135.04395 (C8 H7 O2)	8.63	327	[10]
Phenylethyl primeveroside	434.2017 [M+NH$_4$]$^+$; 439.15684 [M+Na]$^+$	-0.9	295.10193 (C11 H19 O9); 259.08090 (C11 H15 O7); 145.04929 (C6 H9 O4); 133.04944 (C5 H9 O4)	9.67	-	-
*NI	531.07652 [M+H]$^+$; 548.10317 [M+NH$_4$]$^+$	-0.8	163.03886 (C9 H7 O3)	10.35	249; 305; 397	-
*NI	425.10725 [M+H]$^+$; 447.08920 [M+Na]$^+$	-1.4	263.05453 (C13 H11 O6); 217.04912 (C12 H9 O4); 199.03871 (C12 H7 O3); 189.05437 (C11 H9 O3); 173.05945 (C11 H9 O2); 171.04375 (C11 H7 O2); 163.03838 (C9 H7 O3); 161.05952 (C10 H9 O2); 143.04898 (C10 H7 O); 115.05421 (C9 H7)	10.53	372	-
Feruloylquinic acid	369.11763 [M+H]$^+$; 391.09969 [M+Na]$^+$	-1.0	177.05441 (C10 H9 O3); 145.02827 (C9 H5 O2); 117.03364 (C8 H5 O)	10.79	298; 326	-
Myricetin glucoside or isomer	481.0973 [M+H]$^+$	-0.8	319.04419 (C15 H11 O8)	11	-	[2][10]
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_3$)	465.10247 [M+H]$^+$	-0.6	303.04935 (C15 H11 O7)	11.1	342	[3]
$C_{15}H_{10}O_8$ coupled to 2hexoses, 1deoxyhexose, 1coumaric acid	935.24414 [M+H]$^+$; 789.18698 [M+H-deoxyhexose]$^+$	-1.1	481.09668 (C21 H21 O13); 319.04416 (C15 H11 O8); 147.04387 (C9 H7 O2)	11.15	286; 310	-
*NI	749.19212 [M+H]$^+$	-0.3	587.14166 (C28 H27 O14); 425.10718 (C19 H21 O11); 325.09070 (C15 H17 O8); 307.08099 (C15 H15 O7); 263.05450 (C13 H11 O6); 217.04977 (C12 H9 O4); 199.03874 (C12 H7 O3); 163.03871 (C9 H7 O3)	11.51	339	-
Flavonoid-O-hexoside (flavonoid aglycon: $C_{15}H_{10}O_4$)	435.12808 [M+H]$^+$; 457.2.4027 [M+Na]$^+$	-1.1	273.07544 (C15 H13 O5); 255.06500 (C15 H11 O4); 227.06993 (C14 H11 O3); 199.07516 (C13 H11 O2); 153.01813 (C7 H5 O4); 149.02332 (C8 H5 O3)	11.6	-	-
*NI	875.18622 [M+H]$^+$	-1.6	567.09692 (C24 H23 O16); 319.04416 (C15 H11 O8); 147.04387 (C9 H7 O2)	11.64	283; 318	-
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_3$)	465.10247 [M+H]$^+$	-0.6	303.04935 (C15 H11 O7)	12.17	-	[3]
$C_{18}H_{12}O_8$-*O*-hexoside	407.13317 [M+H]$^+$; 429.11503 [M+Na]$^+$	-1.2	245.08055 (C14 H13 O4); 217.08572 (C13 H13 O3); 147.04395 (C9 H7 O2)	12.28	354	-
Isorhamnetin-*O*-hexoside	479.11782 [M+H]$^+$	-1.2	317.065 (C16 H13 O7); 302.04153 (C15 H10 O7)	12.29	-	[3]
*NI	567.09721 [M+H]$^+$	-1.5	-	12.29	-	-
*NI	583.0922 [M+H]$^+$	-1.3	335.039 (C15 H11 O9); 317.02866 (C15 H9 O8); 289.03369 (C14 H9 O7)	12.29	-	-
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_3$)	465.10247 [M+H]$^+$	-0.6	303.04935 (C15 H11 O7)	12.44	341	[3]
Dicaffeoylquinic acid	517.13405 [M+H]$^+$	0.0	163.03876 (C9 H7 O3); 145.02829 (C9 H5 O2); 135.04398 (C8 H7 O2); 117.03352 (C8 H5 O)	13.1	300; 328	[3][10]
Isorhamnetin-*O*-hexoside	479.11767 [M+H]$^+$	-1.5	317.06491 (C16 H13 O7); 302.04153 (C15 H10 O7); 285.03818 (C15 H9 O6); 274.04614 (C14 H10 O6); 257.04370 (C14 H9 O5)	13.37	-	[3]
Dicaffeoylquinic acid	517.13405 [M+H]$^+$	0.0	163.03876 (C9 H7 O3); 145.02829 (C9 H5 O2); 135.04398 (C8 H7 O2); 117.03352 (C8 H5 O)	13.44	300; 328	[3][10]
*NI	551.17521 [M+H]$^+$; 573.15673 [M+Na]$^+$	-1.3	245.08055 (C14 H13 O4); 217.08572 (C13 H13 O3); 147.04395 (C9 H7 O2)	13.74	351	-
Dicaffeoylquinic acid	517.13405 [M+H]$^+$	0.0	163.03876 (C9 H7 O3); 145.02829 (C9 H5 O2); 135.04398 (C8 H7 O2); 117.03352 (C8 H5 O)	13.85	300; 328	[3][10]
Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_3$)	465.10247 [M+H]$^+$	-0.6	303.04929 (C15 H11 O7)	13.92	-	[3]
*NI	691.09231 [M+H]$^+$	-1.0	513.06665 (C24 H17 O13); 307.04434 (C14 H11 O8); 179.03363 (C9 H7 O4); 163.03885 (C9 H7 O3); 161.02310 (C9 H5 O3); 149.02351 (C8 H5 O3); 145.02843 (C9 H5 O2); 135.04401 (C8 H7 O2); 133.02841 (C8 H5 O2); 123.04418 (C7 H7 O2); 117.03374 (C8 H5 O); 105.03374 (C7 H5 O)	14.02	251; 327; 391	-
Dicaffeoylquinic acid methyl ester	531.14914 [M+H]$^+$	-1.1	-	14.07	-	[3]
Methoxyoxalyl-dicaffeoylquinic acid	603.13365 [M+H]$^+$; 620.16000 [M+NH4]$^+$; 625.11521 [M+Na]$^+$	-1.3	423.09119 (C19 H19 O11); 163.03876 (C9 H7 O3); 145.02831 (C9 H5 O2); 135.04395 (C8 H7 O2)	14.17	328	-
Dicaffeoylquinic acid methyl ester	531.14914 [M+H]$^+$	-1.1	-	14.42	-	[3]
*NI	691.09231 [M+H]$^+$	-1.0	321.04019 (C18 H9 O6); 295.06073 (C17 H11 O5); 249.05466 (C16 H9 O3); 181.04984 (C9 H9 O4); 163.03885 (C9 H7 O3); 151.03909 (C8 H7 O3); 145.02843 (C9 H5 O2); 135.04401 (C8 H7 O2); 133.02841 (C8 H5 O2); 123.04418 (C7 H7 O2); 117.03374 (C8 H5 O); 105.03374 (C7 H5 O)	14.45	251; 323; 389	-
*NI	691.09231 [M+H]$^+$	-1.0	295.06073 (C17 H11 O5); 249.05466 (C16 H9 O3); 181.04984 (C9 H9 O4); 163.03885 (C9 H7 O3); 151.03909 (C8 H7 O3); 145.02843 (C9 H5 O2); 135.04401 (C8 H7 O2); 133.02841 (C8 H5 O2); 123.04418 (C7 H7 O2); 117.03374 (C8 H5 O); 105.03374 (C7 H5 O)	14.57	252; 306; 327; 395	-
Helichrysin	449.14537 [M+H]$^+$	2.6	-	14.77	-	[10]
Dicaffeoylquinic acid methyl ester	531.14914 [M+H]$^+$	-1.1	-	14.82	-	[3]
*NI	777.12893 [M+H]$^+$; 794.15571 [M+Na]$^+$	-1.1	:9 (C17 H9 O4); 267.06488 (C16 H11 O4); 265.04950 (C16 H9 O4); 251.06946 (C16 H11 O3); 249.05466 (C16 H9 O3); 233.05965 (C16 H9 O2); 221.05957 (C15 H9 O2); 179.03380 (C9 H7 O4); 163.03885 (C9 H7 O3); 151.03909 (C8 H7 O3); 149.02333 (C8 H5 O3); 145.02843 (C9 H5 O2); 135.04401 (C8 H7 O2); 13	14.96	250; 307; 328; 400	-
Quercetin coupled to coumaric acid and hexose	611.13859 [M+H]$^+$; 633.12037 [M+Na]$^+$	-1.5	303.04938 (C15 H11 O7)	15.03	271; 308	-
*NI	575.17289 [M+Na]$^+$	-1.1	-	15.34	259; 270; 291; 307; 326; 349	
Tiliroside (kaempferol-3-*O*-*p*-coumaroylglucopyranoside)	595.14372 [M+H]$^+$; 617.12527 [M+Na]$^+$	-1.5	309.09637 (C15 H17 O7); 287.05432 (C15 H11 O6); 147.04388 (C9 H7 O2); 119.04924 (C8 H7 O)	15.79	314	[3][7]
*NI	639.19099 [M+H]$^+$; 656.21758 [M+NH$_4$]$^+$; 661.17295 [M+Na]$^+$	-1.5	425.0932 (C15 H21 O14); 283.09711 (C17 H15 O4); 245.08081 (C14 H13 O4); 197.09576 (C14 H13 O); 178.07755 (C14 H10); 169.06465 (C12 H9 O); 152.06187 (C12 H8); 141.06993 (C11 H9); 115.05443 (C9 H7); 101.03880 (C8 H5)	15.94	259; 270; 290; 307; 327; 349	
Isomer of naringenin	273.07509 [M+H]$^+$	-2.4	255.06436 (C15 H11 O4); 227.06993 (C14 H11 O3); 199.07516 (C13 H11 O2); 153.01813 (C7 H5 O4); 149.02332 (C8 H5 O3)	16.52	291	-
Methoxyluteolin	317.06498 [M+H]$^+$	-1.9	302.04141 (C15 H10 O7)	16.58	-	[2]
Isomer of naringenin	273.07509 [M+H]$^+$	-2.4	255.06436 (C15 H11 O4); 227.06993 (C14 H11 O3); 199.07516 (C13 H11 O2); 153.01813 (C7 H5 O4); 149.02332 (C8 H5 O3)	16.76	291	-
Apigenin	271.06010 [M+H]$^+$	0.0	-	17.79	-	[9]
Isorhamnetin	317.06558 [M+H]$^+$	0.0	-	17.91	-	-
Methoxyflavonoid ($C_{16}H_{14}O_5$)	287.0907 [M+H]$^+$	-2.4	271.05826 (C15 H11 O5)	18.5	291; 347	-
4-Hydroxy-3-(3-methyl-2-butenyl) acetophenone	205.12197 [M+H]$^+$	-1.7	149.05954 (C9 H9 O2)	18.66	-	[6]
Pinocembrin	257.0802 [M+H]$^+$	-2.5	215.06953 (C13 H11 O3); 173.05952 (C11 H9 O2); 153.01805 (C7 H5 O4); 131.04909 (C9 H7 O); 103.05442 (C8 H7)	19.08	289	[7]
Gnaphalin A	315.08631 [M+H]$^+$	0.0	300.06229 (C16 H12 O6); 285.03885 (C15 H9 O6); 257.04382 (C14 H9 O5)	19.43	273; 353	[4][6]
Gnaphalin B	315.08631 [M+H]$^+$	0.0	299.05438 (C16 H11 O6); 285.03857 (C15 H9 O6); 282.05103 (C16 H10 O5); 257.04391 (C14 H9 O5); 254.05592 (C15 H10 O4)	19.81	270; 318	[4][6]
Methoxyflavonoid ($C_{17}H_{12}O_5$)	285.07505 [M+H]$^+$	-2.5	270.05139 (C15 H10 O5)	20.06	-	-
Helipyrone	321.1329 [M+H]$^+$	-1.1	167.07018 (C9 H11 O3); 155.07021 (C8 H11 O3)	20.92	-	[5][8]
Arzanol	403.1744 [M+H]$^+$	-1.8	-	21.66	-	[1][5][8]
Methylarzanol	417.1902.4 [M+H]$^+$	-1.3	-	22.03	-	[5]

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CHAPTER 6

UNRAVELLING THE POTENTIAL OF THE MEDICINAL HALOPHYTE *ERYNGIUM MARITIMUM* L.: *IN VITRO* INHIBITION OF DIABETES-RELATED ENZYMES, ANTIOXIDANT POTENTIAL, POLYPHENOLIC PROFILE AND MINERAL COMPOSITION

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Unravelling the potential of the medicinal halophyte *Eryngium maritimum* L.: *In vitro* inhibition of diabetes-related enzymes, antioxidant potential, polyphenolic profile and mineral composition

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ABSTRACT

This work searched for the biotechnological potential and chemical characterisation of the medicinal halophyte *Eryngium maritimum* L. as source of bioactive natural products with enzymatic inhibitory properties and antioxidant capacity. With that aim, herbal formulations traditionally used in folk medicine, namely tisanes (infusions and decoctions) and tinctures, were prepared from four different anatomical organs (roots, stems, leaves and flowers) and assessed for *in vitro* inhibition of enzymes related with diabetes and for antioxidant potential. Phenolic fingerprinting and mineral contents were also assessed. Sea holly's tisanes, particularly from flowers and leaves, had the highest phenolic content although tinctures were comparatively richer considering the ingestion dosage. The main constituents identified were carvacrol, 2,3-dimethoxybenzoic acid, naringenin, catechin and *t*-cinnamic acid; from the several compounds identified, naringin, naringenin and 2,3-dimethoxybenzoic acid are here firstly described in the genus and epicatechin and carvacrol in the species. In all extracts, sodium was the most abundant mineral, followed by potassium, calcium and magnesium, pointing to possible nutritional applications of these beverages/tinctures as macronutrients supplementary source. Sea holly's tinctures (stems, leaves and flowers) were capable of inhibiting dietary carbohydrate digestive enzymes (α-glucosidase and α-amylase) and had *in vitro* antioxidant potential, particularly flowers. Altogether, results highlight that sea holly extracts, especially tinctures from stems, leaves and flowers, could be a novel source of α-glucosidase and α-amylase inhibitors, antioxidant compounds and also phenolic and mineral constituents, thus suggesting they may be interesting to further explore as potential health-promoting herbal beverages, food additives or other products.

1. Introduction

The *Eryngium* genus comprises around 250 species widespread throughout the world (Castroviejo et al., 2003) and, like other members of the Apiaceae family such as fennel, parsley or celery, many *Eryngium* plants have ornamental, culinary or medicinal uses. In traditional medicine, *Eryngium* species have numerous applications namely as diuretic, kidney stone inhibitor, hypoglycaemic, anti-inflammatory, poison antidote, stimulant and antitussive, among many others (Wang et al., 2012; Erdem et al., 2015). *Eryngium maritimum* L., commonly named sea holly, is a halophyte plant typically native to the dunes and sandy beaches of the Mediterranean region, Black Sea, Atlantic and Baltic coasts, and has been introduced into parts of Eastern North America and Australia (Castroviejo et al., 2003; Isermann and Rooney, 2014). Its roots and young shoots are eaten cooked in some Mediterranean regions like Apulia in Italy (Isermann and Rooney, 2014; Guarrera and Savo, 2016). In Mediterranean countries it is a folk remedy used as diuretic, kidney stone inhibitor, aphrodisiac, expectorant, anthelmintic, antitoxin against various infections, for oedema reabsorption and/or pain relief (Lisciani et al., 1984; Isermann and Rooney, 2014; Erdem et al., 2015). Moreover, sea holly has been introduced into cosmetics as cell-suspension cellulosomes in anti-ageing face creams (Rossanalabs, 2018). Research on *E. maritimum* has been conducted mainly on organic extracts and essential oils from either aerial parts or roots, and reports antimicrobial, antioxidant and/or anti-inflammatory activities and main constituents like phenolic acids, flavonoids and terpenes (Lisciani et al., 1984; Meot-Duros et al., 2008; Kholkhal et al., 2012; Wang et al., 2012; Amessis-Ouchemoukh et al., 2014; Darriet et al., 2014; Yurdakok et al., 2014; Erdem et al., 2015; Conea et al., 2016; Mejri et al., 2017; Rjeibi et al., 2017). Few reports focused on aqueous or hydroethanolic extracts namely following the usage given in folk practices (infusions, decoctions and tinctures of the medicinal plant)

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and seldom did studies perform a comparative assessment of the different plant organs.

Herbal medicine has always had a strong presence throughout humankind history, playing a fundamental role in human welfare as a first-line health support for patients with any type of condition, and it still is of utmost importance in many cultures today (Mehta et al., 2015). In fact, due to their great biosynthetic capacity, plants are a valuable source of bioactive compounds (*e.g.* polyphenols) that are responsible for the health benefits of botanical extracts (Mehta et al., 2015; Schmidt et al., 2008). With scientific research attracting the attention to medicinal plants as reservoirs of novel compounds and/or natural products, the demand for these health-promoting herbal commodities continues to increase (Gruenwald, 2009; Mehta et al., 2015). Although many traditional medicinal plants like *Aloe vera* [aloe], *Aloysia citrodora* [lemon verbena], *Camelia sinensis* [tea] or *Aspalathus linearis* [rooibos] are explored by the food industry as beverages or additives for functional foods (Gruenwald, 2009; Melucci et al., 2013; Pohl et al., 2016), medicinal halophytes are typically overlooked despite being great sources of highly bioactive molecules and of potential new-generation products with beneficial properties (Ksouri et al., 2012). Nonetheless, recent scientific endeavours have set a new perspective on these salt-tolerant plants, namely their prospective uses in herbal beverages, like *Crithmum maritimum*, *Helichrysum italicum* subsp. *picardii* or *Limonium algarvense*, or as raw material for different commercial segments and/or industries, like *Polygonum maritimum*, *Artemisia campestris subsp. maritima*, *Lithrum salicaria* or *Juncus* species (Lopes et al., 2016; Rodrigues et al., 2016, 2017a, 2017b; Rodrigues et al., 2017; Pereira et al., 2017a, 2017b, in press). Moreover, different researchers that embraced the current effort to value medicinal plants, halophyte species included, have focused on their *in vitro* capacity to inhibit key-enzymes associated with human health conditions like diabetes, neurodegeneration or skin hyperpigmentation (*e.g. Schisandra chinensis* (Turcz.) Baill., *Euphorbia denticulata* Lam., *Lycium barbarum* L., *Capparis spinosa* L. or halophytes *P. maritimum*, *H. italicum* subsp. *picardii*, *A. campestris* subsp. *maritima*, *J. acutus, L. algarvense*) (Mocan et al., 2016, 2017, 2018; Zengin et al., 2016, 2017; Rodrigues et al., 2017a, 2017b; Rodrigues et al., 2017; Pereira et al., 2017b, in press; Mollica et al., 2018).

It is acknowledged that natural products are in high demand for positive outcomes particularly in anti-ageing, cognitive well-being and/or other effects such as managing diabetes (Gruenwald, 2009). Following these considerations and in addition to the increased demand of raw plant materials for therapeutic purposes, studying medicinal plants, particularly halophytes, bearing in mind their folk use, can reveal new sources of compounds, products or material for different segments like the food, pharmaceutical and/or cosmetic industries. In this context, the current work aims at assessing sea holly from a medicinal use perspective, *i.e.*, focusing on tisanes and tinctures and their respective ingestion dosage (cup-of-tea or drops). Extracts from four different anatomical organs were prepared and assessed for *in vitro* inhibition of carbohydrate digestive enzymes and radical scavenging activity (RSA) and metal-related potential, coupled with a chemical characterisation comprised of phenolic fingerprinting and mineral contents to better characterise the plant material (Melucci et al., 2018). To the best of our knowledge, this is the first study of sea holly in terms of its anti-diabetic and antioxidant properties and as source of phytoconstituents and minerals in the form of common therapeutic dosages of the different organs.

2. Material and methods

2.1. Plant collection

Eryngium maritimum L. (Apiaceae) whole plants were collected in Praia de Faro beach (36°59′50.4″N 7°58′44.4″W), located in the south Portuguese coast, in June of 2015. Dr. Manuel J. Pinto, botanist in the National Museum of Natural History (University of Lisbon, Botanical Garden, Portugal), carried out the taxonomical classification and the herbarium of Marbiotech laboratory keeps a voucher specimen (voucher code MBH37). Plants were divided in roots, stems, leaves and flowers, and they were oven-dried until complete dryness at 45 °C (~3 days), milled and stored at −20 °C until use.

2.2. Extracts preparation

Aqueous extracts were prepared by homogenising 1 g of dried plant material in 200 mL of ultrapure water, similarly to a regular cup-of-tea. The biomass was immersed in boiling water for 5 min to prepare infusions, while for decoctions biomass was boiled in water during 5 min. The hydroethanolic extracts were prepared as a home-made tincture: 20 g of dried plant material was homogenised in 200 mL of 80% aqueous ethanol for seven days. Independent extractions ($n \geq 3$) of the different plant materials were made. Extracts were filtered (Whatman n° 4) and stored in their original form at −20 °C until use. Some aliquots of the extracts were freeze-dried and/or vacuum-dried for yield determination and high-performance liquid chromatography (HPLC-PDA) analysis, and, in the case of tinctures, also to test for bioactivities (dried extracts were re-suspended in aqueous ethanol and tested at a concentration of 10 mg/mL). Samples from the independent extractions were tested for bioactivities, phenolic (spectrophotometric) and mineral content and, since no significant differences were found among corresponding extracts from the different extractions, freeze-dried extracts were pooled accordingly for the HPLC-PDA analysis.

2.3. Phytochemical composition of the extracts

2.3.1. Total phenolic (TPC), flavonoid (TFC) and condensed tannin (CTC) content

TPC, TFC and CTC were estimated by the spectrophotometric methods Folin–Ciocalteu, aluminium chloride colorimetric and 4-dimethylaminocinnamaldehyde (DMACA) assays, respectively, using gallic acid, quercetin and catechin as standards, as described in Rodrigues et al. (2015). Results are presented as milligrammes of standard equivalents (GAE, QE and CE, respectively) per cup-of-tea for infusions and decoctions (mg/200 mL) or per mL for tinctures (mg/1 mL; 1 mL equals approximately 3 to 5 drops, the more common ingestion dosage; Cook, 1896). Further information related to these methods is provided in the Supplementary materials section S1 (Table S1.1).

2.3.2. Phenolic fingerprinting by high-performance liquid chromatography – photodiode array detection (HPLC-PDA)

HPLC analyses were performed on a Waters liquid chromatograph equipped with a solvent pump model 600 and a 2996 photodiode array detector, and data was acquired with Empower v2 Software (Waters Spa, Milford, MA, USA). A C18 reversed-phase packing column (Prodigy ODS(3), 4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA) was used for separation and the column was thermostated at 30 ± 1 °C with a Jetstream2 Plus column oven. The UV/Vis acquisition wavelength was set within the 200–500 nm range and the quantitative analyses were achieved at maximum wavelength for each compound, as reported in the Supplementary materials section S2. The injection volume was 20 μL and the mobile phase was directly *on-line* degassed using Biotech DEGASi mod. Compact (LabService. Anzola dell'Emilia. Italy). Gradient elution was performed using the mobile phase water-acetonitrile (93:7, v/v, 3% acetic acid). Stock solutions of 22 standards (benzoic acid, catechin, carvacrol, *t*-cinnamic acid, chlorogenic acid, *o*-coumaric acid, *p*-coumaric acid, 2,3-dimethoxybenzoic acid, epicatechin, *t*-ferulic acid, gallic acid, harpagoside, *p*-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, naringenin, naringin, quercetin, rutin, sinapinic acid, syringic acid, and vanillic acid) were made at a concentration of 1 mg/mL in a final volume of 10 mL of methanol. Extracted samples were dissolved in the mobile

phase at a concentration of 1 mg/mL. All prepared sample solutions were centrifuged and the supernatant was injected into the HPLC-PDA system. Analytical figures of merit on method validation are reported in literature (Locatelli et al., 2017) and were confirmed herein after evaluation of matrix interferences. Concentrations of identified phenolics were calculated as mg/cup-of-tea (water extracts) or μg/1 mL (tinctures), based on the extracts' yield.

3. Mineral composition

Tisanes and tinctures were analysed directly for mineral content (in appropriately diluted samples) by Microwave Plasma–Atomic Emission Spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), as described in Pereira et al. (2017a). Appropriate blanks were produced and analysed. Limits of quantification (LOQs) were calculated using the lowest calibration point included in the calibration curve (Ca: 0.540, Cd: 0.047, Cr: 0.004, Cu: 0.002, Fe: 0.048, K: 0.467, Mg: 0.146, Mn: 0.001, Na: 0.293, Ni: 0.005, Pb: 0.016, Zn: 0.014 μg/L). Results were expressed as mg or μg per cup-of-tea for tisanes and per mL for tinctures.

3.1. Biological activities

3.1.1. In vitro anti-diabetic activity: inhibition of the enzymes microbial and mammalian α-glucosidases and α-amylase

The capacity of the extracts to inhibit the α-glucosidase enzymes was assessed following Kwon et al. (2008), using acarbose (10 mg/mL) as positive control. The microbial α-glucosidase enzyme was obtained from *Saccharomyces cerevisiae* yeast; an example of a mammalian-origin α-glucosidase enzyme was obtained from a crude extract from rat's intestine acetone powder. The α-amylase inhibitory activity was determined according to the method described in Rodrigues et al. (2017a), using acarbose (10 mg/mL) as positive control. Results were calculated in relation to a control (ultrapure water/aqueous ethanol) and expressed as percentage of inhibitory activity in a cup-of-tea for tisanes and as percentage (%) activity in 10 mg/mL extracts for tinctures.

3.1.2. Antioxidant activity assessed by four radical-based assays

The radical scavenging activity (RSA) of the extracts was assessed against four different radicals (DPPH [1,1-diphenyl-2picrylhydrazyl], ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], NO [nitric oxide], and $O_2^{\cdot-}$ [superoxide]) as described elsewhere (Rodrigues et al., 2015, 2016), using BHT (butylated hydroxytoluene), ascorbic acid and catechin as positive controls. Results were calculated in relation to a control containing ultrapure water or aqueous ethanol and expressed as percentage of antioxidant activity in a cup-of-tea for tisanes and as percentage (%) activity in 10 mg/mL extracts for tinctures.

3.1.3. Antioxidant activity assessed by three metal-related assays

The Fe^{3+} reducing activity (ferric reducing antioxidant power, FRAP) of the extracts and their capacity to chelate copper (CCA) and iron (ICA) were assessed as previously described (Rodrigues et al., 2015), using EDTA (ethylenediaminetetraacetic acid) and BHT as positive controls. Results were calculated in relation to a positive control for FRAP and in relation to a negative control (ultrapure water/aqueous ethanol) for CCA and ICA. They are presented as percentage of antioxidant activity in a cup-of-tea for tisanes and as percentage (%) activity in 10 mg/mL extracts for tinctures.

3.2. Statistical analysis

Experiments were performed at least in triplicate and results are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to assess significant differences ($p < 0.05$) or, when parametricity of data did not prevail, the Kruskal Wallis one-way analysis of variance on ranks; if significant, the respective pairwise multiple comparison tests Tukey or Dunn's were carried out. XLStat® version 19.5 was used to execute statistical analyses.

4. Results and discussion

4.1. Phytochemical fingerprinting

Phenolics are some of the most important plant bioactive metabolites, and herbal extracts, such as tisanes for example, are important sources of these compounds in the human diet (Balasundram et al., 2006). In this context, tisanes and tinctures from *E. maritimum* were assessed spectrophotometrically for their total phenolic (TPC), flavonoid (TFC) and condensed tannin (CTC) contents and results are summarised on Table 1. The polyphenolic contents are presented as milligrammes per cup-of-tea for infusions and decoctions (mg/200 mL), given that the "cup-of-tea" is a worldwide measure for drinking tisanes, or mg per mL for tinctures (mg/1 mL) since 1 mL equals approximately 3 to 5 drops, which is a common ingestion therapeutic dosage (Cook, 1896). When comparing TPC and TFC among all the sea holly's organs and extracts, flowers' herbal teas have higher TPC (14.9–17 mg/200 mL) and leaves' tisanes have higher TFC (1.62–1.85 mg/200 mL). However, given the two different ingestion dosages, tinctures are comparatively richer in TPC (particularly roots, 7.61 mg/mL) and TFC (leaves in particular, 0.61 mg/mL) considering that's their content in 1 mL as opposed to the content in 200 mL (cup-of-tea) of the water extracts. Other authors reported TPC values in sea holly's leaves' methanol extracts between 16 and 44 mg GAE/g dry weight (Meot-Duros et al., 2008; Amessis-Ouchemoukh et al., 2014), which are comparable to those presently obtained in the same organ when considering the corresponding TPC values adjusted to mg/g based on the extraction yields (32–34 mg GAE/g; Table 1). TFC reported by those authors (24 mg QE/g dw) was similar to the current total flavonoids in leaves' hydroethanolic extracts (22 mg QE/g; Table 1) but aqueous extracts had lower TFC. Rjeibi et al. (2017) was the only study found comparing sea holly's organs and using aqueous extracts but the TPC reported (roots 4.3, stems 1.7, leaves 8.1 mg GAE/g dw) was lower than that currently obtained, as was TFC in leaves' extracts (Table 1). However, these authors determined TFC in roots and stems, which, in the present study, was not found. Species-specific factors such as provenance, harvesting and/or environmental characteristics are variables that can influence the synthesis of plants' secondary metabolites,

Table 1
Phenolic contents in infusions, decoctions (mg/cup-of-tea, 200 mL) and tinctures (mg/mL) from *E. maritimum* organs and respective yields (infusion and decoctions: mg extract/200 mL, tinctures: mg extract/mL).

Organ	Extract	Yield	TPC[1]	TFC[2]	CTC[3]
Roots	Infusion	521	3.32 ± 0.29^{f}	<LQ	<LQ
	Decoction	443	3.72 ± 0.30^{f}	<LQ	<LQ
	Tincture	53.6	7.61 ± 0.65^{d}	<LQ	<LQ
Stems	Infusion	214	4.25 ± 0.21^{ef}	<LQ	<LQ
	Decoction	224	5.34 ± 0.40^{e}	<LQ	<LQ
	Tincture	22.6	0.42 ± 0.02^{g}	0.15 ± 0.01^{f}	<LQ
Leaves	Infusion	350	12.0 ± 0.69^{c}	1.85 ± 0.12^{a}	<LQ
	Decoction	348	11.2 ± 0.82^{c}	1.62 ± 0.10^{b}	<LQ
	Tincture	27.7	0.88 ± 0.04^{g}	0.61 ± 0.04^{e}	<LQ
Flowers	Infusion	263	17.0 ± 0.62^{a}	1.19 ± 0.19^{c}	<LQ
	Decoction	268	14.9 ± 0.39^{b}	0.80 ± 0.08^{d}	<LQ
	Tincture	24.9	0.76 ± 0.06^{g}	0.12 ± 0.01^{f}	<LQ

Data represent the mean ± SD ($n \geq 6$). In each column, different letters mean significant differences ($p < 0.05$). LQ: limit of quantification.
[1] TPC: total polyphenol content, mg GAE/200 mL or GAE/mL, GAE: gallic acid equivalents; LQ (TPC) = 4.0 μg GAE/mL.
[2] TFC total flavonoid content; mg QE/200 mL or QE/mL, QE: quercetin equivalents; LQ (TFC) = 6.0 μg QE/mL.
[3] CTC: condensed tannin content, mg CE/200 mL or CE/mL, CE: catechin equivalents; LQ (CTC) = 7.8 μg CE/mL.

reflected in the different phytochemical contents reported (Ksouri et al., 2012; Buhmann and Papenbrock, 2013). Nonetheless, Meot-Duros et al. (2008) considered that *E. maritimum* leaves' extracts have lower phenolic content comparatively to other halophyte species, although possessing bioactive compounds with great potential for food or cosmetic industries. In fact, sea holly's tisanes showed lower phenolic and flavonoid contents (3.32–17.0 mg GAE/cup-of-tea and 0.80–1.85 mg QE/cup-of-tea, Table 1) when compared with herbal teas from other halophyte species already assessed by Pereira et al. (2017a, 2017b), *C. maritimum* (12.4–35.3 mg GAE/cup-of-tea and 22.9–57.2 mg RE/cup-of-tea) and *H. italicum* subsp. *picardii* (13.9–76.5 mg GAE/cup-of-tea and 20.3–119 mg RE/cup-of-tea).

The phytochemical fingerprint of infusions, decoctions and tinctures from sea holly was further explored by HLPC-PDA to identify and quantify individual phenolic compounds in these samples. The described HPLC method is simple, accurate (precise and true) and selective for separation and quantification of phenolics in the considered samples, while reasonably sensitive for direct quantitative determination of these compounds in real matrices. Obtained results (mg/cup-of-tea for water extracts and μg/1 mL for tinctures, calculated based on the extraction yields) are presented in Table 2. Chromatograms obtained from real sample analyses at 278 nm are reported in Supplementary materials section S3. Sixteen phenolic compounds were identified and quantified in sea holly's tisanes and tinctures: nine phenolic acids, five flavonoids and two other constituents (Table 2). Most of these phenolics were already described in *Eryngium* species except for naringin, naringenin and 2,3-dimethoxybenzoic acid which are, to the best of our knowledge, here firstly described in the genus. Moreover, no reports were found detailing epicatechin and carvacrol in *E. maritimum*, which are being here described for the first time in this species.

The phenolic fingerprint of sea holly's organs showed that roots' herbal teas, particularly infusions, had the higher quantity of compounds determined (infusions 25.5 and decoctions 10.5 mg/cup-of-tea), mainly due to the dominant phenolic monoterpene found in this organ, carvacrol. Nevertheless, if considering the two ingestion dosages, tinctures have comparatively higher amounts (particularly roots, 833 μg/mL, and leaves 669 μg/mL). Carvacrol was the major constituent found, the only one identified in all samples and reaching 21 mg/cup-of-tea in roots' infusion. This phenolic monoterpene, more abundant in roots (4.05% of the infusion extract, 0.69% of decoction and 0.37% of tincture extracts; Table 2, considering the yields obtained), has not been reported in *E. maritimum* but is described in *E. bungei* as a major constituent in aerial parts' essential oils (8.9%; Erdem et al., 2015). Carvacrol is mostly known for being the predominant component in the

Table 2
Phenolic fingerprint in infusions, decoctions (mg/cup-of-tea, 200 mL) and tinctures (μg/mL) from *E. maritimum* organs, determined by HPLC-PDA.

Compound (Peak)	Roots			Stems			Leaves			Flowers		
	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture
Phenolic acids												
Hydroxybenzoic acids												
Gallic acid	–	–	67.5 ± 2.7	–	–	–	–	–	–	–	–	–
3-OH-benzoic acid	–	–	–	–	–	–	0.19 ± 0.02	–	–	–	0.11 ± 0.01	9.5 ± 1.0
Vanillic acid	–	–	–	–	–	–	0.13 ± 0.02	–	17.5 ± 0.3	<LOQ	<LOQ	<LOQ
Syringic acid	–	–	–	–	–	–	–	0.17 ± 0.01	–	–	–	–
Hydroxycinnamic acids												
Chlorogenic acid	–	–	–	0.15 ± 0.01	0.11 ± 0.03	4.8 ± 0.5	0.77 ± 0.03	0.69 ± 0.11	16.1 ± 1.1	0.07 ± 0.01	–	–
Sinapinic acid	–	–	–	–	–	–	–	0.07 ± 0.01	–	<LOQ	<LOQ	<LOQ
t-Ferulic acid	0.40 ± 0.04	0.09 ± 0.01	<LOQ	–	–	–	0.18 ± 0.02	0.18 ± 0.02	18.8 ± 1.4	0.07 ± 0.01	0.06 ± 0.01	7.2 ± 1.0
o-Coumaric acid	–	–	–	–	–	<LOQ	0.18 ± 0.02	0.15 ± 0.01	13.3 ± 1.1	0.05 ± 0.01	0.06 ± 0.01	–
t-Cinnamic acid	1.6 ± 1.3	0.19 ± 0.01	28.4 ± 7.5	0.07 ± 0.01	0.10 ± 0.09	–	–	–	8.6 ± 0.8	<LOQ	–	–
Flavonoids												
Flavanols												
Catechin	0.15 ± 0.07	0.47 ± 0.05	12.3 ± 1.0	1.6 ± 0.3	–	186 ± 13	3.34 ± 0.4	1.7 ± 0.2	195 ± 18	0.8 ± 0.5	2.10 ± 0.02	109 ± 12
Epicatechin	–	–	–	–	<LOQ	–	<LOQ	<LOQ	10.8 ± 2.8	<LOQ	0.19 ± 0.03	–
Flavonols												
Rutin	–	–	–	–	–	11.8 ± 2.3	0.32 ± 0.03	–	19.4 ± 0.6	–	–	5.5 ± 0.5
Flavanones												
Naringin	–	–	–	–	–	–	0.11 ± 0.01	0.3 ± 0.2	–	0.10 ± 0.01	0.11 ± 0.01	–
Naringenin	–	3.22 ± 0.70	276 ± 49	1.0 ± 0.4	2.1 ± 0.5	105 ± 51	0.91 ± 0.07	3.6 ± 0.4	274 ± 36	1.2 ± 0.1	1.3 ± 0.3	57.3 ± 5.6
Other constituents												
2,3-DiMeObenzoic acid	2.2 ± 0.2	3.5 ± 0.4	254 ± 47	–	0.07 ± 0.01	–	0.21 ± 0.03	–	23.8 ± 4.4	–	0.11 ± 0.01	29.9 ± 0.01
Carvacrol	21.1 ± 0.4	3.1 ± 0.3	196 ± 67	0.6 ± 0.2	0.8 ± 0.2	37.7 ± 15.1	0.39 ± 0.01	1.2 ± 0.9	71.7 ± 7.6	0.3 ± 0.1	0.5 ± 0.1	42.8 ± 19.7
TOTAL	25.5	10.5	833	3.42	3.14	346	6.78	8.06	669	2.63	4.52	261

Data represent the mean ± SD of 2 different and independent measurements. Limit of quantification (LOQ) = 0.20 μg/mL.

essential oils of *Origanum* and *Thymus* species and has several demonstrated bioactivities, namely proliferative (wound-healing), anti-carcinogenic and anti-mutagenic, analgesic, insecticidal, among others, but its most remarkable one is antimicrobial (Baser, 2008). Considering the levels of this constituent in sea holly's roots infusion, this extract could be an alternative source of carvacrol for the food and feed industries given its potential as flavouring additive with antimicrobial properties (Baser, 2008). Also prominent in roots' extracts was 2,3-dimethoxybenzoic acid (2.2 and 3.5 mg/cup-of-tea in infusion and decoction, 254 μg/mL in tincture, Table 2), here firstly reported in the *Eryngium* genus. Although present in fruits and vegetables, dimethoxybenzoic acids are not very common but some have described antifungal activity concerning fruit and vegetable decay (Lattanzio et al., 1994). Another main constituent determined was naringenin, higher in roots and leaves' decoctions (3.2 and 3.6 mg/cup-of-tea, respectively, Table 2) and also higher in those organs' tinctures (276 and 274 μg/mL, respectively, Table 2). Naringenin was not found described in *Eryngium* species but it is an abundant flavonoid in *Citrus* fruits, particularly grapefruit, where it is reported at a mean concentration of 2.7 mg/100 mL of *C. paradisi* juice (Nakajima et al., 2014; Patel et al., 2014). This value is slightly higher than those presently found in a cup-of-tea (200 mL) of sea holly's decoctions from roots and leaves, but 10 mL of those organs' tinctures would suffice to match the grapefruit's naringenin content. Considering that this flavanone is used in the cosmetic and pharmaceutical industries (Patel et al., 2014), sea holly's extracts seem relevant as an alternative source for this phenolic compound. Moreover, naringenin has shown a promising pharmacological profile with reported antioxidant, anti-inflammatory, anti-cancer, anti-diabetic and anti-obesity activities (Nakajima et al., 2014; Patel et al., 2014). Catechin was also among the main compounds detected, higher in leaves' water extracts (1.7–3.4 mg/cup-of-tea), flowers' decoction and stems' infusion (2.1 and 1.6 mg/cup-of-tea, respectively); in tinctures this flavonoid reached almost 0.2 mg/mL in stems and leaves. Rjeibi et al. (2017) also found higher catechin levels in water extracts from *E. maritimum*'s stems (0.88%) and leaves (0.74%) in values comparable to those herein determined (stems 0.74%, leaves 0.47–0.97%; Table 2, considering the yields obtained). According to Gadkari and Balaraman (2015), cocoa consists of several catechins and a cocoa-product like dark chocolate may contain around 6.6 mg/100 g of catechin, which is only twice as much as a cup-of-tea from sea holly's leaves infusion can supply. The catechin polyphenol group has various biological activities described such as anti-carcinogenic, antioxidant, antidiabetic, protection against degenerative diseases, among others; catechin itself is associated with a decreasing risk of coronary heart disease, antiplatelet effects and anticancer activity (Sutherland et al., 2006; Gadkari and Balaraman, 2015). Yet another main phenolic found was *t*-cinnamic acid in roots' infusion (1.6 mg/cup-of-tea), although at much lower levels or undetected in the other extracts. Rjeibi et al. (2017) have also identified this constituent as a major compound in sea holly's water extracts but at higher concentrations (1.5–3.8%). Besides being described as antioxidant, antitumoural, cytotoxic and antimicrobial (Heleno et al., 2015), cinnamic acid has reported benefits related to its anti-diabetic properties namely lowering of triglycerides levels and improvement of carbohydrate metabolising enzymes, of glucose tolerance and insulin resistance (Alam et al., 2016). The remaining phenolics identified were detected in lower amounts comparatively to the above mentioned main constituents, but chlorogenic acid in leaves tisanes (0.7–0.8 mg/cup-of-tea) and gallic acid in roots tinctures (68 μg/mL) were detected at noteworthy concentrations. In fact, other authors (Mejri et al., 2017; Rjeibi et al., 2017) found these among the major phenolics in *E. maritimum*'s methanol and water extracts from different organs, along with caffeic, protocatechuic and vanillic acids, kaempferol and quercetin, all except vanillic acid not presently determined. Chlorogenic acid has been demonstrated to possess antioxidant, anti-carcinogenic, antimicrobial, hypoglycaemic, hypolipidaemic and hypotensive effects (Meng et al., 2013; Santana-Gálvez et al., 2017), and gallic acid has shown antioxidant, anti-carcinogenic and anti-inflammatory activities (Khadem and Marles, 2010; Ksouri et al., 2012). Altogether, the phenolic fingerprint of sea holly's tisanes and tinctures reveals the presence of compounds described in literature as valuable bioactive phytochemicals, highlighting the potential use of sea holly's extracts as source of bioactive molecules/products. Actually, phenolic compounds are an essential part of the human diet; in edible plant-products they act as pigments, antioxidants, flavour precursors and, as part of our diet, they can be considered health-promoting commodities (Ksouri et al., 2012).

4.2. Mineral composition

Besides possessing a variety of polyphenolic compounds, herbal extracts like tisanes may also be an additional source of other constituents in the human diet such as minerals (Pohl et al., 2016; Pereira et al., 2017a). Hence, the extracts from sea holly's organs were analysed for the presence of these essential nutrients as it could increase their potential value as food products. Table 3 presents the samples' mineral content as mg per cup-of-tea for infusions and decoctions (mg/200 mL) or mg per mL for tinctures (mg/mL), according to the respective common ingestion dosage. Sodium was the mineral more abundantly found in sea holly's extracts, particularly in tisanes from stems (41.6–44.1 mg/cup-of-tea) and leaves (25.3–25.7 mg/cup-of-tea); in tinctures, although in a different dosage, the highest Na levels were also determined in stems (1.5 mg/mL) and leaves (1 mg/mL) extracts. Values of this element in sea holly's stems and leaves herbal teas are similar to those detected in another halophyte's tisanes, the sea fennel *C. maritimum* (20–49 mg/cup-of-tea; Pereira et al., 2017a), although higher than usually found in various regularly consumed tisanes from plants like *Matricaria chamomilla* L. (chamomile) or *Tilia* species (linden) (Pohl et al., 2016). Still, the recommended daily Na intake is 2000 mg maximum (WHO, 2012), hence, a cup-of-tea from any of sea holly's organs is reasonably safe to include in a daily diet although some caution would be advisable due to the cumulative intake of sodium from other food sources. Potassium was also fairly abundant in sea holly's tisanes with levels reaching 20 mg/cup-of tea in flower's decoction and between 16 and 18 mg/cup-of tea in stems and leaves' herbal teas. The sea fennel also showed higher K concentration in flowers' tisanes and in comparable levels (19–23 mg/cup-of-tea; Pereira et al., 2017a). Herbal teas from commonly used plants can range from as little as 2.88 μg/mL up to 852 μg/mL of K (aprox. 170 mg/200 mL; Pohl et al., 2016), which was only surpassed by the K content in sea holly's flowers tincture (913 μg/mL). Of the other macro-elements, calcium was higher in leaves' tisanes (3.6–3.7 mg/cup-of-tea) and magnesium in flowers' decoction (2.3 mg/cup-of-tea) and leaves' herbal teas (2.2 mg/cup-of-tea). In tinctures, these minerals were present in lower concentrations (Ca: 4.1–20 μg/mL, Mg: 20–51 μg/mL) comparatively to Na or K. Comparing to sea fennel's herbal teas, Ca and Mg reached higher maximum levels in that halophyte's leaves decoction (20 and 5.6 mg/cup-of-tea, respectively; Pereira et al., 2017a), although in the remaining organs values were akin to those here reported in sea holly. As for micronutrients, manganese concentration was higher in leaves' tisanes (4.3–4.4 μg/cup-of-tea) while iron and zinc were not found in the aqueous extracts (below LOQ). In tinctures, these microelements' content was below 1 μg/mL. Altogether, our results indicate that sea holly's tisanes and tinctures may contribute to the human daily intake of some elements like Na, K, Ca and Mg (particularly extracts from leaves). Still noteworthy is that Cu and Ni levels were well below reference tolerable upper intake levels (Cu: 10, Ni: 1 mg/day; Otten et al., 2006) and can therefore be considered safe for consumption. Potentially toxic elements like Cr and Cd were not found (below LOQs) and Pb, when detected, was below legislated values for plants (0.3 mg/kg wet weight, maximum levels in finished herbal products are not regulated; EC Regulation, 1881/2006).

Table 3
Mineral content in infusions, decoctions (mg or μg/cup-of-tea, 200 mL) and tinctures (μg/mL) from *E. maritimum* organs.

Mineral	Roots			Stems			Leaves			Flowers		
	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture	Infusion	Decoction	Tincture
Macro-elements												
Na	10.3 ± 0.07^{d}	9.9 ± 0.3^{d}	367 ± 10^{g}	41.6 ± 0.98^{b}	44.1 ± 0.4^{a}	1482 ± 91^{f}	25.3 ± 0.3^{c}	25.7 ± 0.2^{c}	977 ± 62^{fg}	4.19 ± 0.07^{e}	4.9 ± 0.08^{e}	152 ± 10^{g}
Ca	1.77 ± 0.03^{de}	1.76 ± 0.01^{de}	7.7 ± 0.3^{f}	2.25 ± 0.06^{c}	2.5 ± 0.3^{b}	13.7 ± 0.2^{f}	3.58 ± 0.05^{a}	3.71 ± 0.08^{a}	19.7 ± 0.3^{f}	1.57 ± 0.01^{e}	1.83 ± 0.02^{d}	4.1 ± 0.1^{f}
K	10.6 ± 0.2^{e}	9.3 ± 0.1^{f}	500 ± 45^{g}	16.3 ± 1.1^{cd}	17.5 ± 0.07^{b}	736 ± 17^{g}	15.6 ± 0.2^{d}	16.2 ± 0.3^{cd}	677 ± 15^{g}	17.0 ± 0.4^{bc}	20.1 ± 0.02^{a}	913 ± 48^{g}
Mg	0.61 ± 0.05^{g}	0.85 ± 0.01^{f}	19.5 ± 0.5^{h}	1.32 ± 0.01^{e}	1.51 ± 0.03^{d}	44.3 ± 0.4^{h}	2.23 ± 0.04^{b}	2.19 ± 0.03^{b}	50.9 ± 0.3^{h}	1.72 ± 0.02^{c}	2.31 ± 0.01^{a}	45.9 ± 0.7^{h}
Micro and trace-elements												
Fe	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.09 ± 0.01^{a}	<LOQ	<LOQ	0.15 ± 0.03^{a}
Mn	<LOQ	<LOQ	0.002 ± 0.001^{f}	1.37 ± 0.04^{d}	1.52 ± 0.03^{d}	0.03 ± 0.01^{f}	4.3 ± 0.1^{b}	4.4 ± 0.1^{a}	0.10 ± 0.01^{f}	1.74 ± 0.03^{c}	1.11 ± 0.07^{e}	0.03 ± 0.01^{f}
Zn	<LOQ	<LOQ	0.43 ± 0.01^{b}	<LOQ	<LOQ	0.35 ± 0.01^{c}	<LOQ	<LOQ	0.72 ± 0.01^{a}	<LOQ	<LOQ	0.69 ± 0.04^{a}
Cu	8.63 ± 0.06^{a}	8.0 ± 0.3^{a}	0.84 ± 0.02^{e}	4.6 ± 0.3^{c}	5.9 ± 0.1^{b}	0.62 ± 0.01^{e}	0.6 ± 0.5^{e}	<LOQ	0.43 ± 0.01^{e}	1.9 ± 0.3^{d}	0.60 ± 0.03^{e}	0.40 ± 0.01^{e}
Cr	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Ni	5.83 ± 0.06^{b}	6.3 ± 0.2^{a}	0.04 ± 0.01^{e}	2.1 ± 0.1^{d}	1.8 ± 0.2^{d}	0.04 ± 0.01^{e}	2.6 ± 0.2^{c}	2.7 ± 0.2^{c}	0.03 ± 0.01^{e}	2.6 ± 0.2^{c}	2.07 ± 2.05^{d}	0.04 ± 0.01^{e}
Pb	<LOQ	<LOQ	0.17 ± 0.01^{ab}	<LOQ	<LOQ	0.14 ± 0.01^{b}	<LOQ	<LOQ	0.16 ± 0.01^{ab}	<LOQ	<LOQ	0.18 ± 0.03^{a}
Cd	<LOQ	<LOQ	< LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Data represent the mean ± SD ($n = 3$). In each row, different letters mean significant differences ($p < 0.05$).
LOQs: Fe, 9.7 μg/cup-of-tea or 0.05 μg/1 mL; Mn, 0.2 μg/cup-of-tea or 0.001 μg/1 mL; Zn, 2.9 μg/cup-of-tea or 0.01 μg/1 mL; Cr, 0.8 μg/cup-of-tea or 0.004 μg/1 mL; Pb, 3.3 μg/cup-of-tea or 0.02 μg/1 mL; Cd, 9.5 μg/cup-of-tea or 0.05μg/1 mL.

4.3. Biological activities: anti-diabetic (enzyme inhibiting) and antioxidant properties

Numerous plants have long been used in traditional herbal medicine to manage conditions such as type 2 diabetes mellitus (T2DM) (Hung et al., 2012). For example, leaves from another *Eryngium* species, *E. creticum*, are drank in decoction for its hypoglycaemic properties (Yaniv et al., 1987). T2DM is a common metabolic disorder characterised by high plasma glucose levels and, in modern medicine, its management includes inhibiting carbohydrate-hydrolysing enzymes, such as α-glucosidase and α-amylase, therefore retarding carbohydrate digestion and glucose absorption, lowering postprandial blood glucose and reducing hyperglycaemia (Kwon et al., 2008; Kumar et al., 2011). In this context, sea holly's extracts were assessed for their anti-diabetic potential by testing their capacity to inhibit microbial and mammalian α-glucosidases and α-amylase. Results (Table 4) are expressed as percentage of activity in a cup-of-tea for tisanes but as percentage of activity in 10 mg/mL extracts for tinctures. Tinctures were not tested in their ingestion dosage form (crude extracts) like the herbal teas because some tinctures' colouring interfered with the assays and hence had to be diluted. Consequently, results may be underestimating the tinctures real potential in terms of bioactivities while used traditionally. All extracts except roots' tisanes had the capacity to inhibit the microbial α-glucosidase, although with varying degrees, but only tinctures showed activity towards the mammalian α-glucosidase and amylase enzymes. In fact, following a classification to describe the inhibitory activity (%) of plant extracts against enzymes as potent (>50%), moderate (30–50%) and low (<30%) (Vinutha et al., 2007; Custódio et al., 2015), tinctures from stems, leaves and flowers can be considered as potent inhibitors of the three enzymes. However, the tincture was more active towards the microbial α-glucosidase (58–72%) than for its mammalian counterpart (29–55%), a result commonly reported for some compounds/natural extracts (Oki et al., 1999; Rodrigues et al., 2017a; Pereira et al., in press) indicating that enzyme source may influence the extract's inhibitory capacity (Oki et al., 1999). Nevertheless, despite the idea of the mammalian enzyme being a more trustworthy proxy for *in vivo* activity (Oki et al., 1999), aqueous extracts from another *Eryngium*, *E. creticum*, that lacked favourable *in vitro* effectiveness exhibited substantial anti-hyperglycaemic activity in rats (Wang et al., 2012). Moreover, it is also usually reported that natural compounds/extracts have stronger inhibitory activity towards α-glucosidase than against α-amylase (Kwon et al., 2008; Custódio et al., 2015; Rodrigues et al., 2017a) but in the present work, stems and flowers' tinctures had slightly higher activity towards α-amylase. This could be due to particular compounds or to additive/synergistic interactions of compounds present in those extracts. It is increasingly documented that dietary polyphenolic phytochemicals, among multiple biological effects, can

Table 4
Anti-diabetic activity of infusions, decoctions (% activity in a cup-of-tea, 200 mL) and tinctures (% activity in 10 mg/mL extracts) from *E. maritimum* organs: inhibitory activities on microbial and mammalian α-glucosidase enzymes, and on **α-**amylase enzyme.

Samples	Organ	Extract	Microbial α-glucosidase	Mammalian α-glucosidase	α-Amylase
Eryngium maritimum	Roots	Infusion	n.a.	n.a.	n.a.
		Decoction	n.a.	n.a.	n.a.
		Tincture	63.1 ± 2.2^{c}	29.0 ± 2.3^{c}	23.9 ± 1.3^{d}
	Stems	Infusion	21.7 ± 1.6^{i}	n.a.	n.a.
		Decoction	25.7 ± 2.0^{i}	n.a.	n.a.
		Tincture	57.8 ± 1.8^{d}	52.1 ± 3.8^{b}	66.8 ± 2.5^{b}
	Leaves	Infusion	38.8 ± 1.4^{f}	n.a.	n.a.
		Decoction	43.1 ± 0.8^{e}	n.a.	n.a.
		Tincture	72.3 ± 1.4^{b}	50.8 ± 3.4^{b}	58.2 ± 3.9^{c}
	Flowers	Infusion	30.2 ± 1.9^{h}	n.a.	n.a.
		Decoction	33.9 ± 1.4^{g}	n.a.	n.a.
		Tincture	62.6 ± 0.8^{c}	55.0 ± 6.7^{b}	66.5 ± 3.4^{b}
Acarbose*			85.0 ± 0.3^{a}	96.0 ± 0.3^{a}	82.2 ± 3.9^{a}

Values represent the mean ± SD of at least three experiments in triplicate (n = 9). n.a.: not active (% activity lower than 20%). *Positive control, tested at 10 mg/mL.
In each column, different letters mean significant differences ($p < 0.05$).

also modulate enzymatic activity like inhibiting glucosidase and amylase activities and therefore contribute to T2DM management by reducing post-prandial hyperglycaemia (Kumar et al., 2011; Hung et al., 2012). The stronger enzymatic inhibition exerted by stems, leaves and flowers tinctures could be related to their main constituents, namely naringenin, reported to improve hyperglycaemia among other anti-diabetic activities (Nakajima et al., 2014; Patel et al., 2014), catechin, which has known activity against T2DM and hypoglycaemic properties (Sutherland et al., 2006; Meena et al., 2017), and carvacrol, previously described as α-glucosidase and α-amylase inhibitor (Govindaraju and Arulselvi, 2018). Moreover, other phenolics found in sea holly's extracts also have described hypoglycaemic properties, namely chlorogenic and ferulic acids (Meng et al., 2013; Alam et al., 2016), and inhibitory activity towards the digestive enzymes, like chlorogenic, vanillic and gallic acids (Kumar et al., 2011; Williamson, 2013). Overall, these results suggest that sea holly's tinctures, particularly from stems, leaves and flowers, contain compounds capable of inhibiting dietary carbohydrate digestive enzymes and hence could be beneficial in controlling glucose levels and aid in managing T2DM. To the best of our knowledge this is the first time that sea holly is associated to any kind of anti-diabetic properties.

In T2DM, oxidative stress by overproduction of reactive oxygen species (ROS) has been implicated as a causative factor in the induction and progression of the condition, as in several other degenerative alterations like vascular and neurodegenerative diseases, inflammation or carcinogenesis (Saeidnia and Abdollahi, 2013; Panigrahy et al., 2016). Antioxidant phytochemicals are well recognised for effectively fighting ROS thus preventing oxidative damage and reducing or ameliorating oxidative-stress related disorders; they are also positively associated in managing T2DM (Saeidnia and Abdollahi, 2013; Sindhi et al., 2013). Therefore, the antioxidant potential of the sea holly's extracts was assessed, using seven methods targeting RSA and metal-related potential (Table 5). Just like for enzyme inhibiting activities, results are expressed as percentage (%) of activity in a cup-of-tea for tisanes and as percentage (%) of activity in 10 mg/mL extracts for tinctures, which could possibly underestimate the real antioxidant potential of crude tinctures. The classification previously used to describe the inhibition of the carbohydrate digestive enzymes can also be applied to the extracts' antioxidant activity. Overall, tinctures showed potent (>50%) antioxidant capacity except against the NO radical and for iron chelation while infusions and decoctions from leaves and flowers varied between moderate (30–50%) and potent (>50%) antioxidant activity. Flowers' tinctures had consistently the higher activities, closely followed by leaves' tinctures, in scavenging DPPH, ABTS and $O_2^{\cdot-}$ radicals, in reducing iron (FRAP) and in chelating copper. Flowers' infusion was equally effective as its tincture in scavenging DPPH and this organ's tisanes had the highest NO scavenging capacity among extracts. On the other hand, roots, stems and leaves' decoctions were the only beverages able to qualify as potent iron chelators. Other studies have reported on the strong antioxidant properties of different types of extracts from *E. maritimum* (Kholkhal et al., 2012; Amessis-Ouchemoukh et al., 2014; Darriet et al., 2014; Yurdakok et al., 2014; Mejri et al., 2017; Rjeibi et al., 2017), but very few analysed more than one organ. Only Amessis-Ouchemoukh et al. (2014), Rjeibi et al. (2017) and Yurdakok et al. (2014) compared different plant parts: the first two described higher RSA towards DPPH and NO in leaves' methanol and water extracts, respectively, while the later depicted similar results in scavenging DPPH between roots and aerial-parts' water extracts. However, flowers, whose extracts showed the best antioxidant results in the present study, were not mentioned in literature as analysed separately and comparatively to the other organs. Furthermore, medicinal plant studies seldom focus on bioactivities associated to the ingestion dosage of either tisanes (cup-of-tea) or tinctures (drops) and this was the first such attempt with sea holly extracts, as was the use of a comprehensive and complementary battery of seven antioxidant assessing *in vitro* assays. The present results confirm sea holly's *in vitro* antioxidant potential, particularly from flowers, thus showing that hydro-alcoholic extracts and beverages from this plant could be useful in preventing oxidative-stress related disorders. Additionally, as oxidative stress has been considered a mediator in diabetes (Saeidnia and Abdollahi, 2013; Panigrahy et al., 2016), the tinctures combined antioxidant and anti-diabetic potential could reduce oxidative stress and help control glucose levels, benefiting T2DM patients and those at risk of developing the condition.

Many studies in literature credit the extracts' antioxidant activity to their polyphenolic content (Ksouri et al., 2012) but some authors did not find that association (Kähkönen et al., 1999). Accordingly, flowers' tisanes had higher TPC but roots' tinctures were comparatively richer in total phenolics, which does not totally correspond to the antioxidant results obtained. Nevertheless, it is important to mention that colorimetric assays give a TPC estimate and may not incorporate all the phyto-antioxidants in the extract (Tawaha et al., 2007). The presence and amount of individual phenolics (Table 2) in the flowers' extracts, especially tinctures, may contribute for its antioxidant superiority although no particular phenolic compound was found exclusively or at higher concentrations in flowers' tinctures except for 3-hydroxybenzoic acid (9.5 μg/mL, Table 2). Yet, this compound is described as having weak antioxidant activity since the number of

Table 5
Antioxidant activity of infusions, decoctions (% activity in a cup-of-tea, 200 mL) and tinctures (% activity in 10 mg/mL extracts) from *E. maritimum* organs: radical scavenging on DPPH, ABTS, NO and $O_2^{\cdot-}$ radicals, ferric reducing antioxidant power (FRAP) and metal-chelating activities on copper (CCA) and iron (ICA).

Samples	Organ	Extract	Antioxidant activity						
			DPPH	ABTS	NO	$O_2^{\cdot-}$	FRAP	CCA	ICA
Eryngium maritimum	Roots	Infusion	31.7 ± 2.3^{g}	n.a.	43.9 ± 3.4^{c}	n.a.	32.9 ± 2.0^{f}	34.4 ± 3.3^{g}	38.4 ± 3.9^{de}
		Decoction	31.5 ± 1.4^{g}	n.a.	41.8 ± 2.0^{c}	n.a.	30.6 ± 0.9^{f}	37.0 ± 1.7^{fg}	50.3 ± 5.1^{bc}
		Tincture	51.3 ± 3.1^{e}	43.2 ± 1.2^{c}	n.a.	23.3 ± 1.9^{f}	54.1 ± 4.4^{d}	51.0 ± 3.6^{e}	n.a.
	Stems	Infusion	27.6 ± 1.7^{g}	n.a.	33.4 ± 2.1^{d}	n.a.	23.3 ± 1.5^{g}	35.7 ± 2.6^{fg}	33.5 ± 2.5^{ef}
		Decoction	28.4 ± 1.8^{g}	na.a	32.5 ± 2.3^{d}	23.1 ± 1.6^{f}	23.4 ± 0.9^{g}	37.4 ± 3.5^{fg}	54.9 ± 4.3^{b}
		Tincture	68.1 ± 5.1^{c}	94.9 ± 3.9^{b}	n.a.	77.0 ± 2.2^{b}	100 ± 0.01^{a}	64.1 ± 2.7^{d}	22.5 ± 1.2^{g}
	Leaves	Infusion	58.3 ± 2.3^{d}	32.5 ± 2.8^{d}	53.7 ± 2.9^{b}	38.5 ± 3.9^{e}	57.8 ± 1.7^{d}	37.1 ± 3.1^{fg}	43.1 ± 3.9^{cd}
		Decoction	39.1 ± 2.6^{f}	29.0 ± 0.9^{d}	50.1 ± 1.6^{b}	52.9 ± 3.5^{c}	43.0 ± 1.7^{e}	34.5 ± 3.0^{fg}	54.5 ± 5.6^{b}
		Tincture	84.2 ± 4.5^{b}	99.7 ± 0.7^{a}	n.a.	75.6 ± 1.8^{b}	100 ± 0.01^{a}	73.7 ± 1.3^{c}	27.0 ± 1.2^{fg}
	Flowers	Infusion	92.6 ± 0.4^{a}	40.9 ± 3.4^{c}	54.3 ± 3.9^{b}	47.2 ± 1.4^{d}	72.3 ± 2.9^{b}	37.9 ± 2.7^{fg}	24.7 ± 4.4^{g}
		Decoction	84.3 ± 3.1^{b}	28.5 ± 0.9^{d}	53.1 ± 3.8^{b}	54.8 ± 1.9^{c}	66.0 ± 2.8^{c}	39.9 ± 3.7^{f}	n.a.
		Tincture	93.6 ± 0.2^{a}	99.5 ± 1.0^{a}	n.a.	73.6 ± 2.8^{b}	100 ± 0.01^{a}	82.6 ± 1.5^{b}	21.3 ± 1.0^{g}
BHT*			95.7 ± 0.1^{a}	99.6 ± 0.3^{a}			–		
Ascorbic acid*					94.0 ± 0.4^{a}				
Catechin*						91.1 ± 0.5^{a}			
EDTA*								98.4 ± 0.9^{a}	95.0 ± 0.1^{a}

Values represent the mean ± SD of at least three experiments in triplicate (n = 9). n.a.: not active (% activity lower than 20%). *Positive controls, tested at 10 mg/mL.
In each column, different letters mean significant differences ($p < 0.05$).

hydroxyl groups bound to the aromatic ring seems to influence the antioxidant capacity of phenolic compounds and 3-hydroxybenzoic acid only has one (Sroka and Cisowski, 2003). Possible additive/synergistic effects of phytoconstituents in the extract could contribute to its higher antioxidant activity, for example between naringenin and catechin, both known antioxidants (Sutherland et al., 2006; Patel et al., 2014), or between other compounds not identified in the present study. As already mentioned, other authors also found some major phenolics in *E. maritimum*'s extracts not presently determined, such as caffeic and protocatechuic acids, kaempferol or quercetin (Mejri et al., 2017; Rjeibi et al., 2017), which attests to the hypothesis that non-identified constituents could be at play. Still noteworthy is the fact that, globally, extracts with higher enzyme inhibitory capacity were also the more potent antioxidants, namely tinctures from stems, leaves and flowers, an association previously described by Kwon et al. (2008) where eggplant α-glucosidase and α-amylase inhibitory capacities were proportional to scavenging-related antioxidant activity, confirming a possible association between antioxidant and carbohydrate-hydrolysing enzymes inhibitory activities.

5. Conclusions

This work is pioneer in describing the biotechnological assessment of a medicinal halophyte, *E. maritimum*, in terms of its anti-diabetic and antioxidant potential and as source of phytoconstituents and minerals in the form of common therapeutic dosages (cup-of-tea for tisanes, drops for tinctures) of the different anatomical organs. Results highlight the potential role of sea holly's herbal teas and tinctures in managing T2DM and help preventing oxidative-stress related diseases, particularly tinctures from stems, leaves and flowers. More precisely, these extracts could be a novel source of phenolic and mineral constituents, antioxidants and inhibitors of dietary carbohydrate digestive enzymes to be further explored as potential health-promoting herbal beverages, food additives or other products for the food, cosmetic and/or pharmaceutical industries. Still, *in vivo* studies should be pursued to fully explore this plant's therapeutic benefits.

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Conflict of interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2018.06.013.

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SUPPLEMENTARY MATERIALS

SECTION S1: Total phenolic, flavonoid and condensed tannin content parameters

SECTION S2: HPLC-PDA analytical method parameters

SECTION S3: Chromatograms at 278 nm

SECTION S1: Total phenolic, flavonoid and condensed tannin content parameters

Table S1.1 Parameters pertained to the quantification of total polyphenol content (TPC), total flavonoid content (TFC) and condensed tannin content (CTC): calibration curve equations, linearity, detection limits and limits of quantification.

Phenolic group	Calibration curve equation	Linearity	Detection limits (mg/mL)	Limit of quantification (mg/mL)
TPC[1]	y = 1.2534x + 0.0592	R^2 = 0.9976	0.001	0.004
TFC[2]	y = 3.4394x + 0.1132	R^2 = 0.9940	0.002	0.006
CTC[3]	y = 1.0453x + 0.1385	R^2 = 0.9857	0.002	0.008

[1] mg GAE/mL, GAE: gallic acid equivalents
[2] mg QE/mL, QE: quercetin equivalents
[3] mg CE/mL, CE: catechin equivalents

SECTION S2: HPLC-PDA analytical method parameters

Preparation of standard solutions

The stock solutions of twenty standards were made at the concentration of 1 mg/mL in a final volume of 10 mL of methanol. Working solutions of mixed standards at the concentrations of 0.20, 0.5, 1, 2.5, 5, 10, and 20 μg/mL were made by dilution of stock solution in volumetric flasks with the mobile phase. Then the standards were injected into the HPLC-UV/Vis system.

Chromatographic separation

A C18 reversed-phase packing column (Prodigy ODS(3), 4.6 × 150 mm, 5 μm; Phenomenex. Torrance, CA, USA) was used for the separation and the column was thermostated at 30 ± 1°C using a Jetstream2 Plus column oven and a gradient elution using a mobile phase of water-acetonitrile (93:7, *v/v*, 3% acetic acid) was selected, as reported in **Table S2.1**. **Figure S2.1** illustrates the separation of the mixture containing the phenolics standards at 278 nm (wavelength where all compounds are detectable). A robust baseline separation was achieved in 60 minutes (**Figure S2.1**). Retention times and other calibration parameters are reported in **Table S2.2**.

Table S2.1 Gradient elution profile.

Time (min)	% water (3% acetic acid)	% acetonitrile (3% acetic acid)	Flow (mL/min)
0	93	7	1
0.1	93	7	
30	72	28	
38	75	25	
45	2	98	
47	2	98	
48	93	7	
58	93	7	

Table S2.2: Calibration parameters, maximum wavelength and retention times.

Phenolic standard	Slope	Intercept	r^2	Wavelength	Ret. time
Gallic acid	44126	1005	0.9858	271 nm	4.99
Catechin	7022	972.4	0.9361	278 nm	13.36
Chlorogenic acid	22136	2475	0.9650	324 nm	14.29
p-Hydroxybenzoic acid	108088	-774.6	0.9504	256 nm	14.71
Vanillic acid	51494	1141	0.9665	260 nm	17.31
Epicatechin	8841	838.9	0.9542	278 nm	17.87
Syringic acid	21596	369.6	0.9672	274 nm	18.30
3-Hydroxybenzoic acid	17280	-1815	0.9541	295 nm	19.41
3-Hydroxy-4-methoxybenzaldehyde	60154	-187.5	0.9882	275 nm	22.08
p-Coumaric acid	96213	4217	0.9416	309 nm	22.65
Rutin	35896	1420	0.9429	256 nm	25.38
Sinapinic acid	498115	-85290	0.9737	324 nm	26.18
t-Ferulic acid	63520	-1361	0.9969	315 nm	27.75
Naringin	22064	-769	0.9964	285 nm	29.78
2,3-Dimethoxybenzoic acid	7163	-579.3	0.9592	299 nm	30.36
Benzoic acid	12500	842.4	0.9901	275 nm	31.20
o-Coumaric acid	111357	-4293	0.9944	276 nm	34.81
Quercetin	74660	-6774	0.9837	367 nm	40.57
Harpagoside	48111	1000000	0.9921	280 nm	45.49
t-Cinnamic acid	168680	-6103	0.9933	276 nm	45.87
Naringenin	40329	305.8	0.9577	290 nm	46.74
Carvacrol	91780	-25903	0.9896	275 nm	49.95

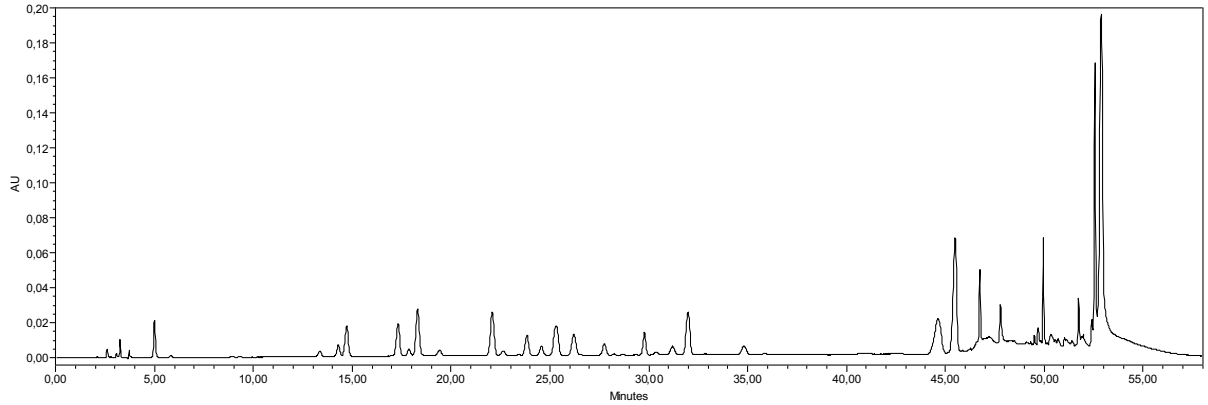
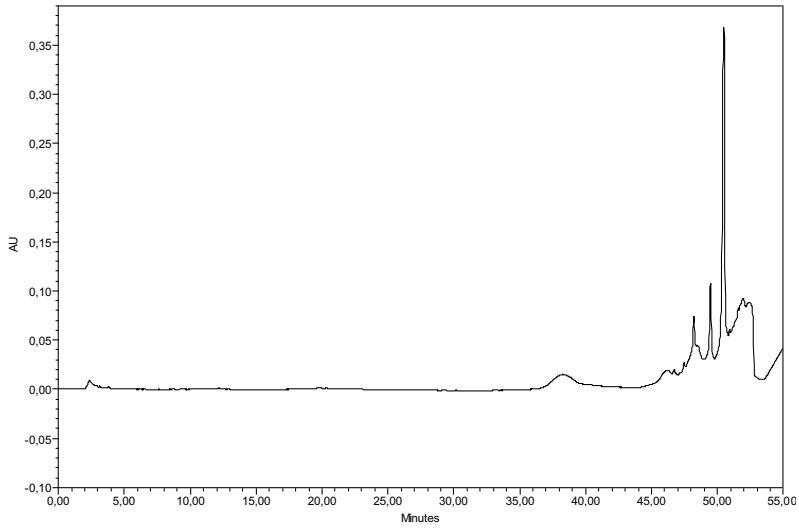


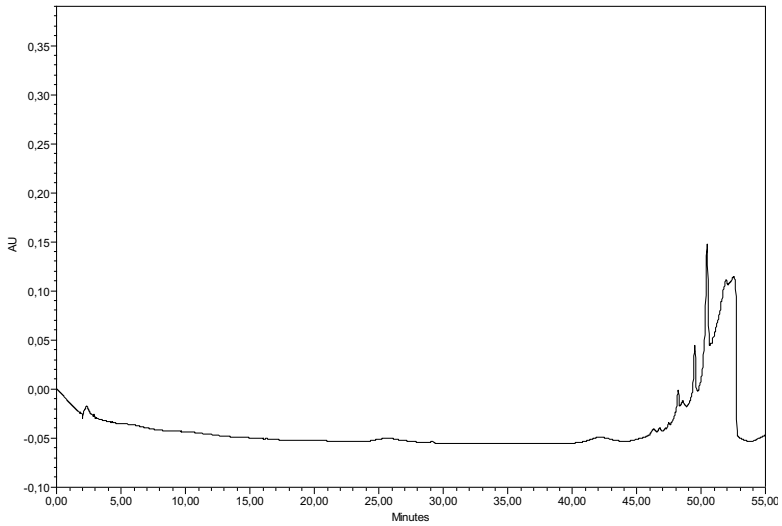
Figure S2.1: Chromatogram of phenolics standards illustrating the separation of standards' mixture (gallic acid, catechin, chlorogenic acid, *p*-hydroxy-benzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin dehydrate, harpagoside, *t*-cinnamic acid, naringenin, carvacrol; wavelength, 278 nm; flow-rate, 1 mL/min; injection volume, 20,0 µL; 4 µg/mL each).

SECTION S3: Chromatograms at 278 nm

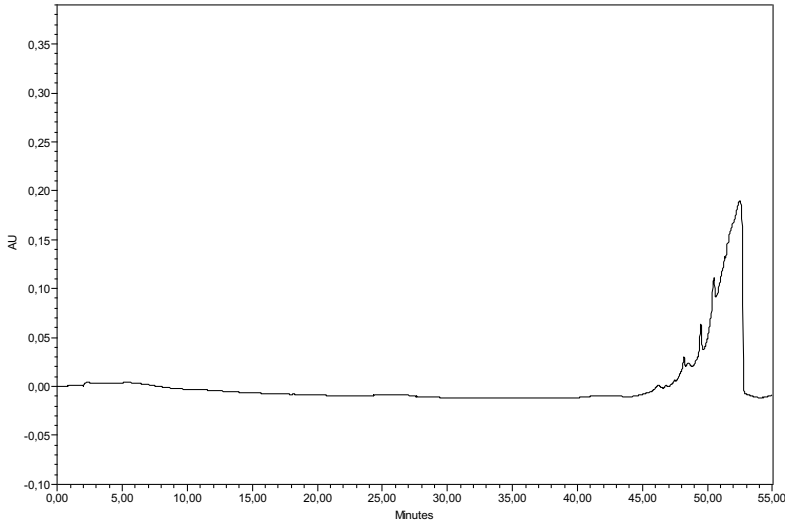
Eryngium maritimum roots infusion



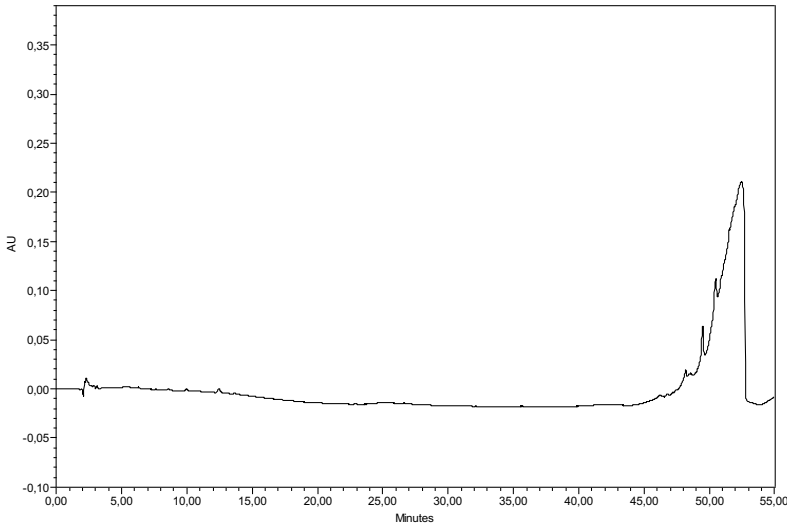
Eryngium maritimum roots decoction



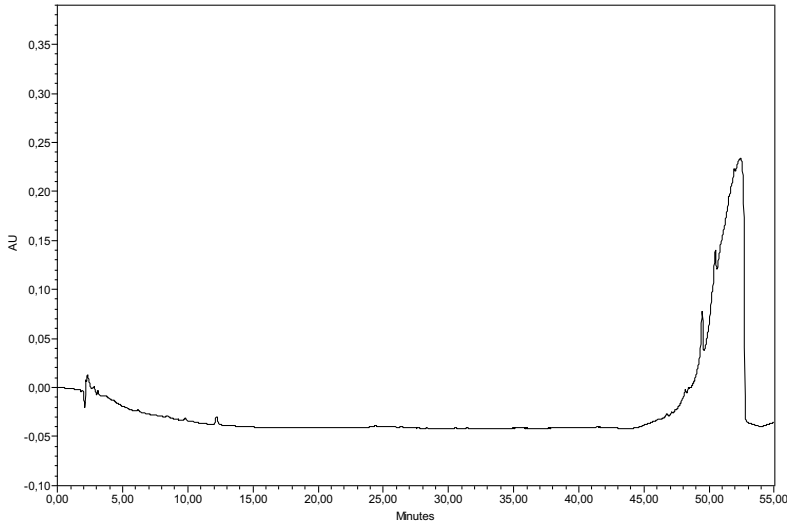
Eryngium maritimum roots tincture



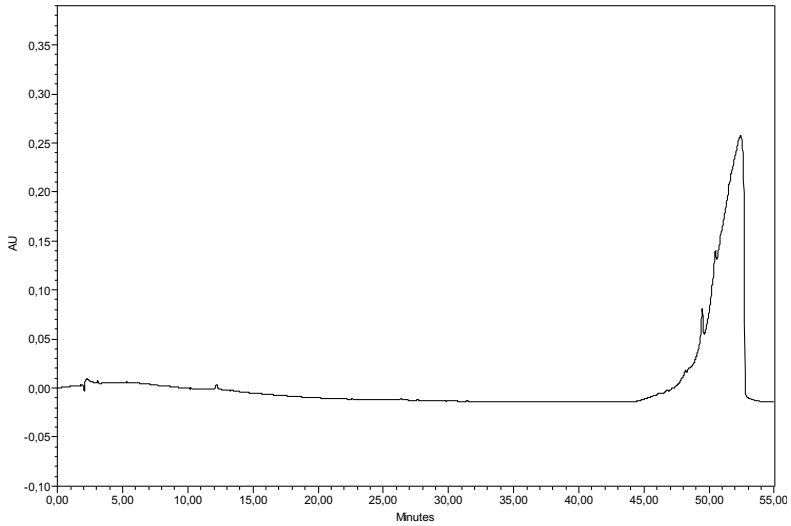
***Eryngium maritimum* stems infusion**



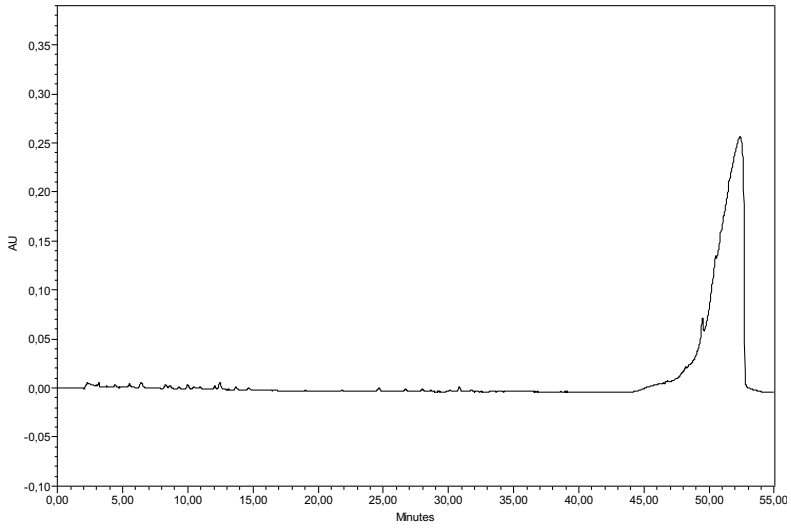
***Eryngium maritimum* stems decoction**



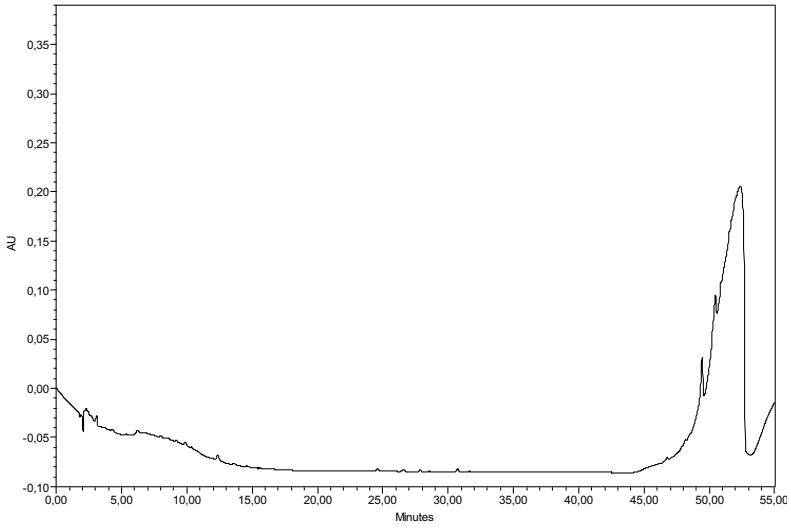
***Eryngium maritimum* stems tincture**



***Eryngium maritimum* leaves infusion**



***Eryngium maritimum* leaves decoction**



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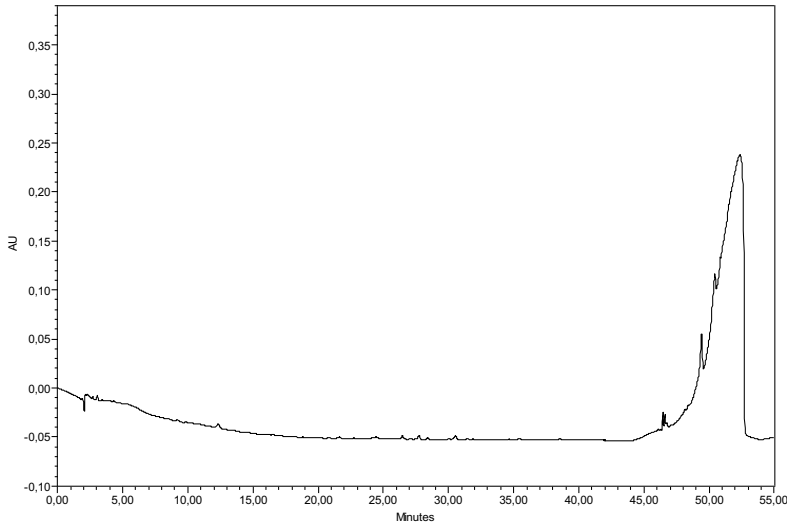
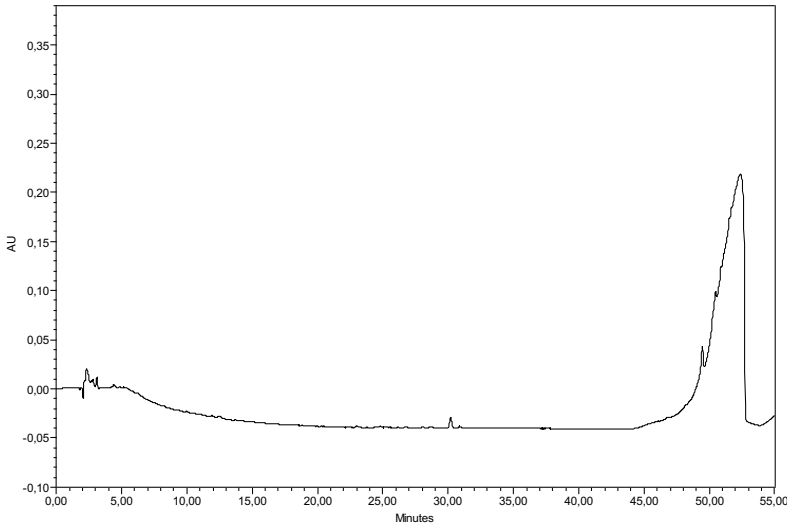
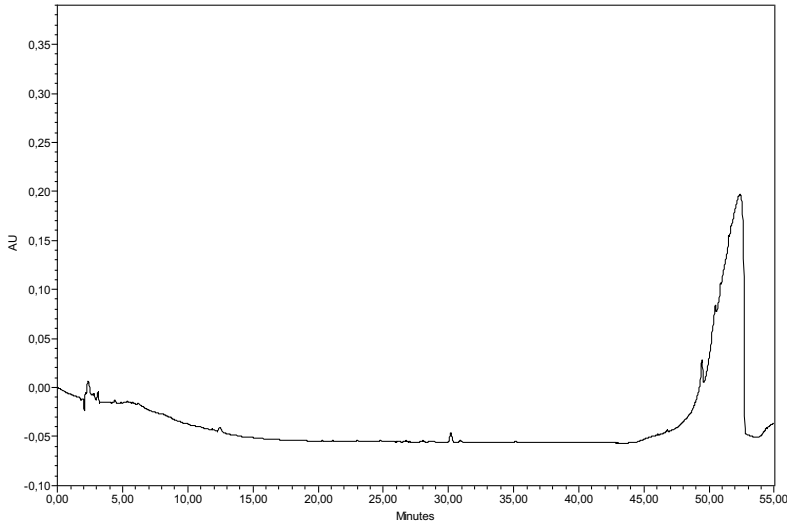


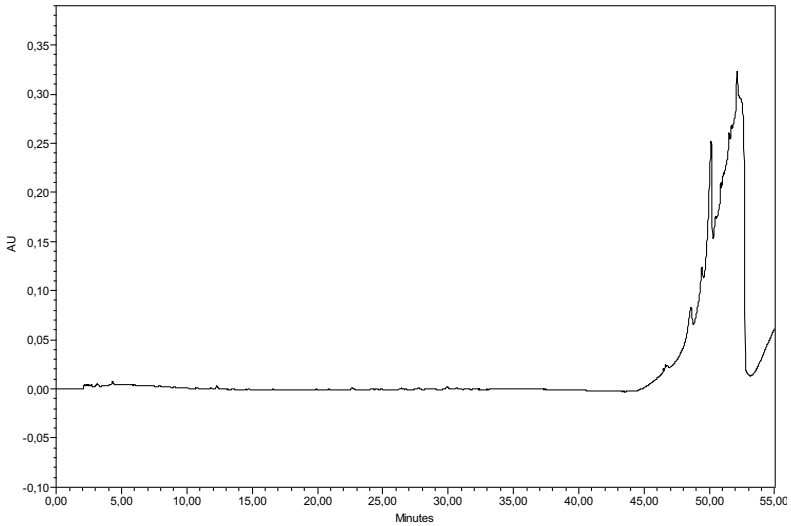
***Eryngium maritimum* flowers infusion**



***Eryngium maritimum* flowers decoction**



***Eryngium maritimum* flowers tincture**



CHAPTER 7

AROMATIC MARINE HALOPHYTES AGAINST *TRYPANOSOMA CRUZI*

Pereira, C.G., Moraes, C.B., Franco, C.H., Panciera, M., Roque, C., Barreira, L., Rodrigues, M.J., Custódio, L. **2018**. Aromatic marine halophytes against *Trypanosoma cruzi*. Marine Drugs (***in prep.***).

Aromatic marine halophytes against *Trypanosoma cruzi*

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Abstract

Marine halophytes are an outstanding reservoir of natural products and / or molecules and many species have ethnopharmacological uses as antimicrobial and / or anti-helminthic. However, little is known of the halophytes' potential against neglected tropical diseases, as for example Chagas disease. This work evaluated for the first time the *in vitro* anti-*Trypanosoma cruzi* activity of natural extracts from the aromatic and medicinal species *Helichrysum italicum* subsp. *picardii* (everlasting) and *Crithmum maritimum* (sea fennel). For that purpose, decoctions, tinctures and essential oils from everlasting's flowers and sea fennel's stems, leaves and flowers were tested against intracellular amastigotes of two *T. cruzi* strains. From the 12 extracts tested one was active, namely sea fennel's flowers decoction, with significant anti-trypanosomal activity and no toxicity towards the host cell (EC_{50} = 17.7 μg/mL, selectivity index > 5.65). This extract was characterized in terms of phenolic profile by high performance liquid chromatography – diode array detection (HPLC-DAD) and the major compounds identified were hydroxycinnamic acids, particularly chlorogenic acid. This sample was then fractionated using liquid-liquid extraction affording 5 fractions, all re-tested in the same model of anti-parasitic activity. Fraction 1 was the most active and selective (EC_{50} = 0.47 μg/mL, selectivity index = 59.6) and was tentatively characterized by liquid chromatography – high resolution mass spectrometry (LC-HRMS). However, the major compound present, and likely responsible for the observed anti-trypanosomal activity, was not successfully identified. A characterization of this fraction by nuclear magnetic resonance (NMR) is currently underway and further research will confirm whether the major compound present is the active molecule. To the best of our knowledge, this is the first report of sea fennel's *in vitro* anti-trypanosomal activity.

Keywords: Chagas disease, marine halophytes, *Trypanosoma cruzi*, *Crithmum maritimum*

7.1. Introduction

Neglected tropical diseases (NTDs) are a group of disabling and chronic infections that flourish primarily in impoverished environments impairing the lives of over one billion people worldwide (Hotez 2007; WHO 2010). Among them is Chagas disease (CD), a potentially life-threatening zoonosis caused by the protozoan *Trypanosoma cruzi*. CD is mainly vector-borne, transmitted to humans through contact with faeces / urine of triatomine bugs (kissing bugs), but can also be transmitted by ingestion of contaminated foods, congenital transmission (mother to foetus), blood transfusion and organ transplants. Traditionally confined to Central and South America, CD is a health and socioeconomic burden that due to growing population movements has spread to other continents and is now an emergent global epidemic with around 6 - 7 million people infected worldwide (Pérez-Molina and Molina 2018; WHO 2017; Zingales 2017). CD involves acute and chronic phases, being more often diagnosed in the chronic stage as the acute infection is typically asymptomatic. In the acute phase, trypomastigotes circulate in the blood (parasitaemia) and infect cells, where they transform into asexually-multiplying amastigotes. When the amastigotes-containing cell is broken, parasites are released and infect other cells. An intense inflammatory response with activation of the innate immune response controls the parasite, and after 4 to 8 weeks parasitaemia decreases substantially. The acute stage usually resolves spontaneously leaving patients chronically infected, if untreated. In the chronic phase, parasites reach and establish in target organs, forming amastigote nests. This stage progresses slowly, and most chronic patients have no further evidence of the disease. After several years, 30 – 40% of chronic patients will develop potentially fatal organ involvement (cardiomyopathy, megaviscera). Recent evidence shows that tissue damage is a result from *T. cruzi* action and the chronic inflammatory response it elicits (Bermudez et al. 2016; Pérez-Molina and Molina 2018; Zingales 2017). Currently only two anti-parasitic drugs are approved for CD treatment, namely nifurtimox and benznidazole, and their efficacy, although high at the acute stage onset, is low in the chronic phase. Moreover, they are rather toxic showing several side effects and require prolonged administration (Bermudez et al. 2016; WHO 2017; Zingales 2017).

Notwithstanding the recent successes in the struggle to eradicate some NTDs, including CD, achieved by specific WHO strategies (e.g. disease monitoring, vector control and preventive chemotherapy; WHO 2010, 2017), the development of new, effective, safe and affordable drugs for CD remains an urgent need (Pérez-Molina and Molina, 2018; Zingales, 2017). With this in mind, researchers have (re)turned to nature to identify new anti-parasitic compounds. For example, the 2015 Nobel Prize of Physiology and Medicine was awarded to

three scientists for their contribution in the field of anti-parasitic drugs, namely the discovery of avermectin from *Streptomyces avermitilis* and of artemisinin from *Artemisia annua* L. These compounds established new therapies to treat lymphatic filariasis, onchocerciasis and malaria, reinforcing the value of natural resources in finding new or alternative drugs (Efferth et al. 2015).

Marine halophytes, a specialized group of plants able to thrive in saline environments, have evolved several adaptations in response to the osmotic and ionic challenges of living in such harsh conditions, including the synthesis of highly bioactive molecules. They represent an outstanding reservoir of natural compounds with some species being used in folk medicine as anti-parasitic and anti-helminthic (Ksouri et al. 2012). However, reports on their potential use against NTDs like CD are scarce (López et al. 2015; Oliveira et al. 2016). *Crithmum maritimum* L. (sea fennel) and *Helichrysum italicum* (Roth) G. Don subsp. *picardii* (Boiss & Reuter) Franco (everlasting) are two aromatic halophytes with described anti-infective uses, namely anti-helminthic and anti-mycotic, and validated antimicrobial activities (Atia et al. 2011; Viegas et al. 2014). In this context, this work evaluated for the first time the *in vitro* anti-trypanosomal properties of decoctions, tinctures and essential oils from both halophytes, namely against the intracellular amastigotes of two *T. cruzi* strains. The active extract was also chemically characterized in terms of phenolic composition by high performance liquid chromatography – diode array detection (HPLC-DAD). The most active extract was further fractionated and all fractions were re-tested; the most promising fraction was tentatively characterized by liquid chromatography – high resolution mass spectrometry (LC-HRMS) to identify the major compound(s) likely responsible for the anti-trypanosomal activity.

7.2. Materials and Methods

7.2.1. Chemicals

All chemicals were of analytical grade. Culture media were purchased from Welgene, Inc. (South Korea), fetal bovine serum (FBS) and penicillin / streptomycin from Gibco Inc. (Life Technologies, USA) and DRAQ5™ from BioStatus Ltd. (UK). Benznidazole was synthesized by Epichem Pty Ltd. (Australia). Commercial standards were supplied by Sigma-Aldrich (Germany). Additional reagents / solvents were obtained from VWR International (Belgium).

7.2.2. Sample collection

Everlasting plants (Asteraceae family; voucher code MBH32) were collected in Ria Formosa coastal lagoon, south Portugal, in Cabanas de Tavira (37°07'51.3"N 7°36'35.6"W) in June 2013, while sea fennel plants (Apiaceae family; voucher code MBH33) were collected in Aljezur beach, in Alentejo coast (37°20'30.7"N 8°51'06.0"W) in August of 2013. Botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal) performed the taxonomical classification. Voucher specimens are kept in the herbarium of XtremeBio's laboratory (CCMAR, University of Algarve, Portugal). Sea fennel plants were divided in stems, leaves and flowers, while only flowers from the everlasting were used. Plant material was oven-dried for 3 days at 40°C until complete dryness, powdered and stored at -20°C until needed.

7.2.3. Preparation of the extracts

Water extracts were prepared similarly to decoctions, by boiling 1 g of dried biomass for 5 minutes in 200 mL of ultrapure water. Hydro-ethanolic extracts were prepared similarly to tinctures, by homogenising 20 g of dried biomass in 200 mL of 80% aqueous ethanol during a week. Extracts were filtered (Whatman nº 4), vacuum and / or freeze-dried and stored in a cool and moist-free environment. To obtain the essential oils (EO), fresh biomass (500 – 1000 g, depending on biomass availability) was cut in small pieces and subjected to hydro-distillation in a Clevenger-type apparatus for 3 hours; EOs were dried with sodium sulphate, filtered, weighed and stored in sealed glass vials at -20°C until further use.

7.2.4. Fractionation of the active extract

After a primary screening of the extracts' anti-trypanosomal activity (described in section 7.2.5), the active extract, sea fennel's decoction from flowers, was fractionated: a 500 mL decoction was prepared and subjected to a sequential liquid-liquid extraction using solvents of increasing polarity (hexane, dichloromethane, chloroform and ethyl acetate; fractions **1** to **4**, respectively). All fractions, including the water residue (fraction **5**), were vacuum concentrated and / or freeze-dried and stored until assessment for anti-trypanosomal activity in a secondary screening (described in section 7.2.5).

7.2.5. Evaluation of *in vitro* anti-trypanosomal activity

All mammalian cell lines, namely human osteosarcoma, U2OS, and *Macaca mulatta* kidney epithelial, LLC-MK2, cells, previously available in C.B. Moraes laboratory (Brasil), were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in humid atmosphere (5% CO_2, 37ºC). LLC-MK2 cultures maintained the *T. cruzi* mammalian cycle *in vitro* and these tissue-derived trypomastigote forms were used to infect U2OS cells in the anti-trypanosomal assay. Two *T. cruzi* strains corresponding to two of the six discrete typing units (DTUs; Sylvio X10/1 strain, DTU I; Y strain, DTU II) were chosen pragmatically based on stocks available at the time the study started. *In vitro* culture of *T. cruzi* was performed as described in Moraes et al. (2014).

Extracts or fractions were dissolved in 100% DMSO either at 5, 10 or 20 mg/mL (according to the different saturation points) and the positive control compound benznidazole was dissolved at 40 mM in DMSO. The anti-trypanosomal assays were executed following Moraes et al. (2014), in duplicate (2 independent experiments); plates were fixed, and parasite and host cell DNA were stained with DRAQ5T^M for microscope imaging (high content screening imaging system, Operetta, Perkin Elmer). Data was analysed according to Moraes et al. (2014): acquired images were analysed with high content analysis software (Harmony, Perkin Elmer) to detect host cell cytoplasm boundary, host cell nucleus and *T. cruzi* DNA, which in turn were quantified to determine total number of cells, number of infected cells, ratio of infected cells, and average number of parasites per infected cell. Only intracellular parasites were scored. Values for ratio of infected cells (infection ratio) were normalized to the average ratio of infected cells from all negative (infected, non-treated cells) and positive (non-infected cells) controls to obtain normalized activity; average cell ratio was determined by the ratio between total cells number in a test well and average total cells number in negative control wells. A primary single-concentration screening was carried out with the extracts (100 μg/mL final concentration, 200 μM for benznidazole) to assess normalized activity (percentage of infection ratio reduction) and average cell ratio (extracts cytotoxicity). The active extract, and afterwards fractions **1** to **5** (see section 2.4), were subjected to a secondary confirmatory dose-response screening, following a 2-fold serial dilution (10 points, 100 μg/mL as the highest concentration tested), with *T. cruzi* Y strain (only strain yielding results for the active extract). Normalized activity datasets were fitted in dose-response curves using GraphPad Prism® to determine EC_{50} (concentration that reduces the infection in 50%), CC_{50} (concentration that

reduces the number of host cells in 50%), selectivity index (CC_{50}/EC_{50}), and maximum activity (max. infection inhibition).

7.2.6. Chemical analysis

As the active extract, sea fennel's decoction from flowers was characterized in terms of phenolic composition by HPLC-DAD. The extract was dissolved at a concentration of 10 mg/mL in ultrapure water and analyzed by HPLC–DAD (Agilent 1100 Series LC system, Germany), equipped with vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A) and a diode array detector (G1315B). Analyses were carried out on a mediterranea sea 18 column (15 × 0.21 cm, 5 µm particle size; Teknokroma, Spain). The mobile phase consisted on a mixture of methanol (solvent A) and 2.5% acetic acid aqueous solution, following a gradient of 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, with 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 (for quantification), 320 and 350 nm. Compounds' concentrations were calculated using calibration curves prepared for each commercial standard (4-hydroxybenzaldehyde, apigenin, catechin hydrate, epicatechin, epigallocatechin, epigallocatechin gallate, pyrocatechol, quercetin, and caffeic, caffeoylquinic, chlorogenic, coumaric, ferulic, gallic, gentisic, p- hydroxybenzoic, neochlorogenic, rosmarinic, salicylic, syringic and vanillic acids) dissolved in methanol at 1.00 mg/mL and diluted to the required concentrations in ultra-pure water. Data acquisition and instrumental control were performed with LC3D ChemStation software (Agilent Technologies). Results were calculated as µg/mg extract dry weight (DW).

From the fractionated extracts (described in section 7.2.4), the active and selective fraction **1** was further tentatively characterized by a liquid chromatography – high resolution mass spectrometry (LC-HRMS) method developed for the characterisation of apolar phytochemicals, to determine its major constituent(s). For LC-HRMS analysis, 1.25 µL of extract was injected with a CTC PAL™ autosampler (CTC Analytics, Switzerland) on a Waters Acquity UPLC HSS C18 SB column (2.1 mm × 100 mm, 1.8 µm; Waters) and thermostatically (35°C) eluted with an Accela™ quaternary solvent manager and a 'Hot Pocket' column oven (Thermo Fisher Scientific). The mobile phase solvents consisted of 50:22.5:22.5:5 (v:v:v:v) water + 5 mM ammonium acetate:methanol:acetonitrile:ethyl acetate (A) and 50:50 (v:v) acetonitrile:ethyl acetate (B); the gradient was set as follows (min/A%): 0.0/90, 0.1/90, 0.8/70,

20.0/9, 20.1/0, 22.4/0, 22.5/90, 25.0/90. For detection, a HRMS (Q Exactive™, Thermo Fisher Scientific) was used: analysis was performed using data dependent fragmentation (ddMS2) in positive and negative ionization mode with heated electrospray ionization (HESI) and atmospheric pressure chemical ionization (APCI). During analysis with both HESI and APCI, ddMS2 data were acquired in positive and negative ionization mode (one analysis per mode) over a mass range (m/z) of 120-1800 to obtain structural information. With HESI, spray voltage was set at ±2.5 kV, sheath gas and auxiliary gas at 47 and 15 (adimensional), respectively, and capillary temperature at 350°C; with APCI, the corona discharge current was set at 5 μA, the vaporizer and capillary temperatures were set at 450°C. For both HESI and APCI, resolving power of 140,000 and 17,500 at full width at half maximum (FWHM) at m/z 200 was used in full MS mode and ddMS2 mode, respectively (stepped collision energy 10, 30, 50 V, isolation window: 6 m/z, isolation offset: 2 m/z).

7.3. Results

Dried biomass was extracted with water (decoctions) and 80% aqueous ethanol (tinctures) while fresh biomass was used to extract EOs, resulting in higher yields (Table 7.1) for sea fennel's water extracts, particularly leaves (45.7%) and flowers (37.8%), and for everlasting's tinctures (32.6%). EOs yields were lower reaching 0.53% for sea fennel's flowers.

To assess the *in vitro* anti-trypanosomal properties of sea fennel and everlasting samples (decoctions, tinctures and EOs) against intracellular amastigotes of two *T. cruzi* strains, a primary single-concentration screening was carried out. Results for normalized activity (Table 7.1), which gives the percentage of infection inhibition in relation to controls, point to 5 promising extracts with ≥ 50% activity against the *T. cruzi* Y strain and 4 extracts against the *T. cruzi* Sylvio X10/1 strain. However, when cross-checking with average cell ratio (Table 7.1), an indicator of extracts' cytotoxicity toward host cells and that should desirably be ≥ 0.5, only sea fennel's flowers decoction covers both parameters and only for the Y strain (65% activity, 0.73 cell ratio). This active extract was subjected to a confirmatory dose-response screening with the *T. cruzi* Y strain and its anti-parasitic activity was corroborated (Table 7.2, Fig. 7.1): an EC_{50} of 17.7 μg/mL and almost 90% maximum activity indicate potency and efficacy against the parasite, and with no toxicity towards the host cell detected within the tested concentrations (if CC_{50} is not determined, the highest tested concentration is used to estimate the SI).

Table 7.1 Extraction yields and primary screening of anti-parasitic activity of *C. maritimum* and *H. italicum* subsp. *picardii* extracts against two *T. cruzi* strains. Normalized activity indicates infection inhibition and average cell ratio indicates extracts' cytotoxicity towards the host cells.

Plant / Compound	**Organ**	**Extract**	**Yields**	***T. cruzi* Y strain**		***T. cruzi* Sylvio X10/1 strain**	
				Normalized activity (%)	Average cell ratio	Normalized activity (%)	Average cell ratio
C. maritimum	Stems	Decoction	29.2%	-1.42 ± 4.50	0.79 ± 0.05	-1.66 ± 5.74	1.36 ± 0.19
		Tincture	20.8%	68.1 ± 10.0	0.14 ± 0.06	70.2 ± 16.8	0.13 ± 0.01
		Essential oils	0.23%	0.00 ± 0.00	0.01 ± 0.01	55.7 ± 27.0	0.13 ± 0.11
	Leaves	Decoction	45.7%	13.6 ± 3.47	0.47 ± 0.05	11.8 ± 3.26	0.52 ± 0.23
		Tincture	26.5%	35.2 ± 4.07	0.26 ± 0.11	33.2 ± 12.2	0.38 ± 0.06
		Essential oils	0.30%	75.1 ± 13.1	0.02 ± 0.02	22.2 ± 18.9	0.02 ± 0.02
	Flowers	Decoction	37.8%	65.0 ± 6.04	0.73 ± 0.04	29.3 ± 0.69	2.00 ± 1.47
		Tincture	32.4%	35.4 ± 31.5	0.01 ± 0.00	73.3 ± 49.5	0.01 ± 0.00
		Essential oil	0.53%	107 ± 0.47	0.00 ± 0.00	12.0 ± 69.7	0.02 ± 0.02
H. italicum subsp. *picardii*	Flowers	Decoction	27.8%	-5.76 ± 1.51	0.12 ± 0.03	-10.6 ± 9.18	0.32 ± 0.07
		Tincture	32.6%	13.4 ± 8.36	0.09 ± 0.01	-7.68 ± 3.23	0.13 ± 0.01
		Essential oil	0.30%	76.1 ± 15.3	0.37 ± 0.14	93.4 ± 7.04	0.36 ± 0.24
Benznidazole[a]				99.5 ± 0.45	1.82 ± 0.16	98.6 ± 0.62	2.11 ± 0.62

Data represent the mean ± SD of two independent experiments/each strain.

[a] Positive control.

Being the most active and least cytotoxic extract, sea fennel's flowers decoction was then fractionated by liquid-liquid extraction using hexane, dichloromethane, chloroform and ethyl acetate, and all fractions (including the water residue) were evaluated for anti-trypanosomal activity against *T. cruzi* Y strain. Results (Table 7.3, Fig. 7.2) show that the hexane fraction (fraction **1**) was the most active and selective, presenting higher potency (EC_{50} = 0.47 µg/mL) and efficacy (113% max. activity), least cytotoxicity (CC_{50} = 28.0 µg/mL) and higher selectivity towards the host cells (SI = 59.6). The positive control benznidazole had comparatively lower potency (EC_{50} = 0.92 µg/mL) while similar efficacy and similar or higher selectivity (109% max. activity, SI > 56). Still noteworthy, fraction **2** (dichloromethane) exhibited high efficacy against the parasite (97% max. activity) and moderate selectivity (SI > 6.47), with an EC_{50} slightly lower and therefore more potent than that of the crude extract (fraction 2, EC_{50} = 12.3 µg/mL, Table 7.3; sea fennel's flowers decoction, EC_{50} = 17.7 µg/mL, Table 7.2).

Table 7.2 Dose-response screening of anti-parasitic activity of the active extract, sea fennel's flowers decoction, against *T. cruzi* Y strain.

Extract / Compound	***T. cruzi* Y strain**			
	EC_{50}[b]	Max. activity[c] (%)	CC_{50} (µg/mL)[d]	SI[e]
Active extract	17.7 ± 1.38 µg/mL	89.4	ND	> 5.65
Benznidazole [a]	3.97 ± 0.93 µM	100	ND	> 101

Data represent the mean ± SD of two independent experiments. ND: not determined

[a] Positive control (3.97 µM = 1.03 µg/mL); [b] EC_{50} is a measure of potency; [c] Maximum activity is a measure of efficacy against the parasite; [d] CC_{50} is a measure of cytotoxicity towards host cells; [e] SI indicates extract/compound selectivity towards the parasite.

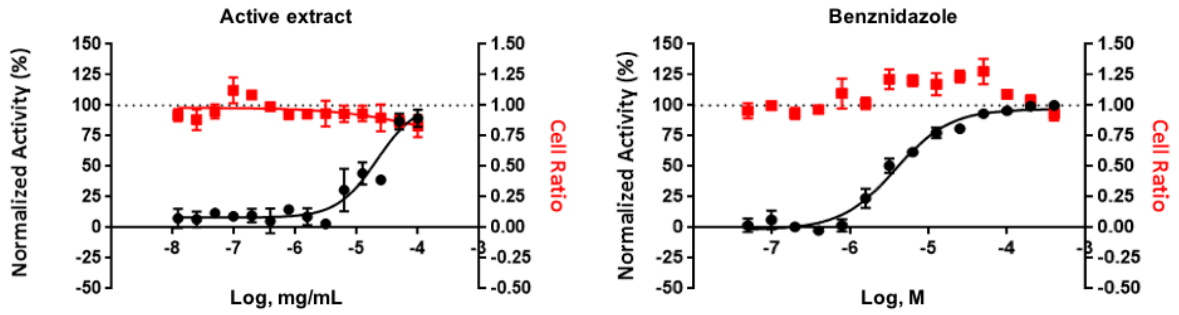

Figure 7.1 Anti-parasitic activity of the active extract, sea fennel's flowers decoction, and of the control benznidazole: dose-response curves normalized to infected and non-infected controls. Data refers to mean values of at least two independent experiments. Black dots refer to normalized activity while red dots refer to cell ratio.

Table 7.3 Dose-response screening of anti-parasitic activity of the active extract's fractions **1** to **5** (hexane, dichloromethane, chloroform, ethyl acetate, water) against *T. cruzi* Y strain.

Extract / Compound	***T. cruzi* Y strain**			
	EC_{50} (µg/mL)[b]	Max. activity[c] (%)	CC_{50} (µg/mL)[d]	SI[e]
Fraction **1**, Hex	0.47 ± 0.01	113	28.0 ± 0.90	59.6
Fraction **2**, Dcm	12.3 ± 0.35	97.0	79.3*	> 6.47
Fraction **3**, Clor	23.3*	56.6	ND	> 4.29
Fraction **4**, Acet	ND	39.4	ND	-
Fraction **5**, H_2O	ND	42.0	ND	-
Benznidazole[a]	0.92 ± 0.02	109	ND	> 56

Data represent the mean ± SD of two independent experiments. ND: not determined
Hex: hexane, Dcm: dichloromethane, Clor: chloroform, Acet: ethyl acetate, H_2O: water
[a] Positive control (3.97 µM = 1.03 µg/mL); [b] EC_{50} is a measure of potency; [c] Maximum activity is a measure of efficacy against the parasite; [d] CC_{50} is a measure of cytotoxicity towards host cells; [e] SI indicates extract/compound selectivity towards the parasite; *Values obtained in one experiment.

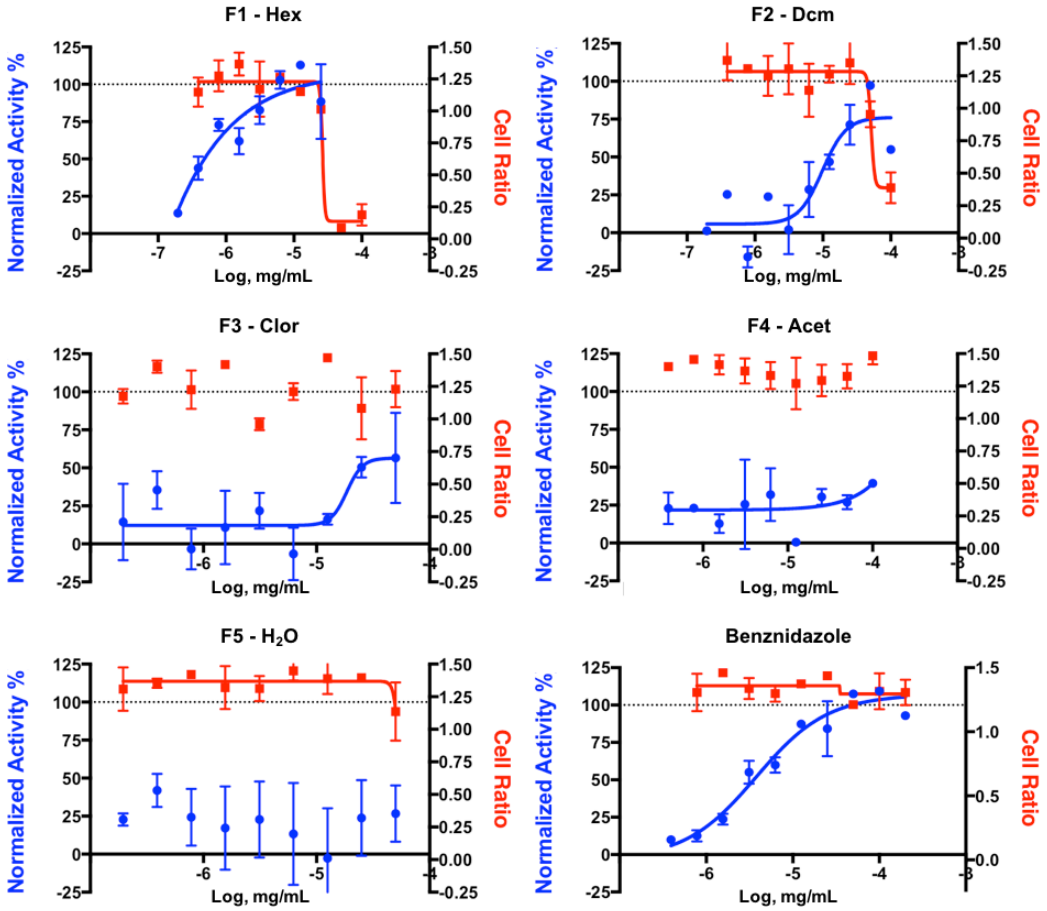

Figure 7.2 Anti-parasitic activity of the active extract's fractions (F1 - Hex, hexane; F2 - Dcm, dichloromethane; F3 - Clor, chloroform; F4 - Acet, ethyl acetate; F5 - H_2O, water), and of the control benznidazole: dose-response curves normalized to infected and non-infected controls. Data refers to mean values of at least two independent experiments. Blue dots refer to normalized activity while red dots refer to cell ratio.

The phenolic composition of the active extract, sea fennel's decoction from flowers, was investigated by HPLC-DAD, aiming at the identification of individual phenolic compounds. Table 7.4 (and Fig. 7.3) shows 10 polyphenolics were identified and quantified. The extract is composed of mainly hydroxycinnamic acids (13.7 out of 14.9 mg/g extract DW) with chlorogenic acid as the dominant compound (9.40 mg/g extract DW), followed by crypto and neochlorogenic acids (1.65 and 1.10 mg/g extract DW, respectively). Epicatechin was the only flavonoid found, at almost 1 mg/g extract (0.9 mg/g extract DW). However, it should be mentioned that non-identified peaks in the chromatogram (Fig. 7.3) indicate the presence of other (non-identified) compounds.

Table 7.4 HPLC–DAD analysis of the phenolic profile (mg/g extract DW) of the active extract, *C. maritimum*'s flowers decoction. Peak numbers refer to the compounds in Figure 7.3.

Compound	Peak nº	RT (min)	Extract
Phenolic acids			
Hydroxybenzoic acids			
Gallic acid	1	1.5	< LOQ
p-Hydroxybenzoic acid	2	4.4	0.12
Hydroxycinnamic acids			
Neochlorogenic acid	3	2.8	1.10
Cryptochlorogenic acid	4	7.4	1.65
Chlorogenic acid	5	7.8	9.40
Coumaric acid	6	11.6	0.79
Ferulic acid	7	13.0	0.72
Flavonoids			
Flavanols			
Epicatechin	8	10.5	0.90
Other polyphenols			
Pyrocatechol	9	2.5	0.21
4-Hydroxybenzaldehyde	10	5.0	< LOQ
TOTAL			**14.9**

RT – retention times; LOQ – Limit of quantification = 0.01 mg compound/g extract dw

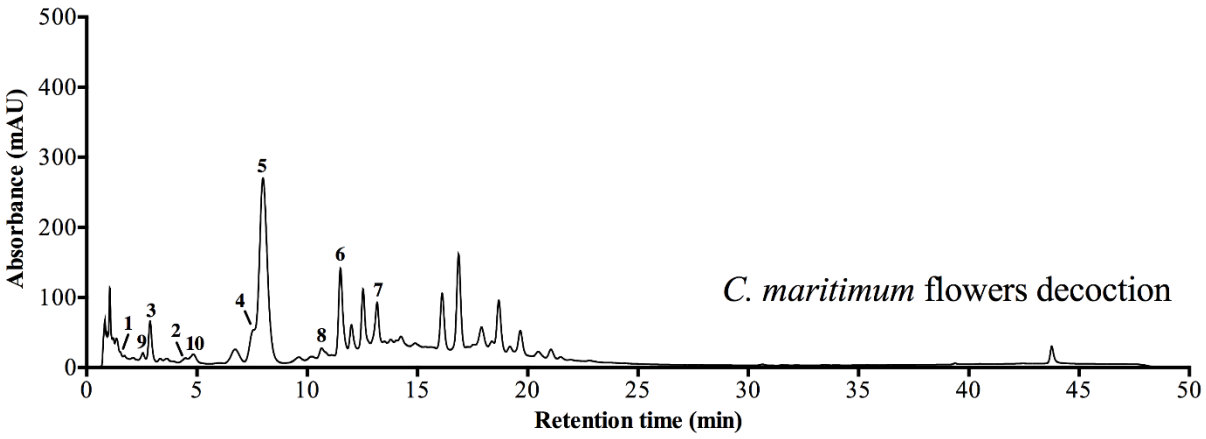

Figure 7.3 HPLC-DAD analysis of phenolic compounds in the active extract, *C. maritimum*'s flowers decoction: chromatogram at 280 nm. Peak numbers refer to the compounds in Table 7.4.

The active and selective fraction 1 (hexane fraction from the active extract) was tentatively characterized by a LC-HRMS method developed for the characterisation of non-polar phytochemicals. Based on the LC-MS analysis, there is one main compound present in that fraction but, unfortunately, the spectral information was inconclusive and the compound was not identified (Table 7.5). Several other compounds were detected in considerately lower intensity; they are most probably similar in structure, judging from the fragmentation data, and detected in less amounts (data not shown). Other low abundant compounds were tentatively identified as fatty acids (data not shown).

Table 7.5 LC-HRMS analysis of the active fraction 1 (hexane): spectral data of the main compound present (but not identified).

	Accurate mass of precursor ion	Molecular Formula	Mass deviation (ppm)	Fragmentation Mass	Molecular formula		
APCI pos	663.4546	$C_{43}H_{60}N_4P$	-0.574	551.32805	$C_{34}H_{48}O_4P$	or	$C_{35}H_{44}N_4P$
		$C_{42}H_{64}O_4P$	1.442	495.26613	$C_{30}H_{40}O_4P$		$C_{31}H_{36}N_4P$
				439.20258	$C_{26}H_{32}O_4P$		$C_{27}H_{28}N_4P$
				383.13969	$C_{22}H_{24}O_4P$		$C_{23}H_{20}N_4P$
				327.07876	$C_{18}H_{16}O_4P$		$C_{19}H_{12}N_4P$
				251.04654	$C_{12}H_{12}O_4P$		$C_{13}H_8\ N_4P$
				233.03576	$C_{12}H_{10}O_3P$		-
				153.07003	$C_{12}H_9$		$C_{12}H_9$
				91.05416	C_7H_7		C_7H_7
APCI neg	473.28375	$C_{29}H_{38}N_4P$	-0.437	457.25234	$C_{27}H_{38}O_4P$	or	$C_{28}H_{34}N_4P$
		$C_{28}H_{42}O_4P$	2.389	441.22127	$C_{26}H_{34}O_4P$		$C_{27}H_{30}N_4P$
				267.11595	$C_{14}H_{20}O_3P$		$C_{21}H_{15}$
				251.08463	$C_{13}H_{16}O_3P$		$C_{20}H_{11}$
				205.15989	$C_{14}H_{21}O$		$C_8H_{22}N_4P$
				189.12847	$C_{13}H_{17}O$		$C_7H_{18}N_4P$
				175.11284	$C_{12}H_{15}O$		$C_6H_{16}N_4P$
HESI pos	663.45438	$C_{43}H_{60}N_4P$	-0.951	Same as for APCI			
		$C_{42}H_{64}O_4P$	1.065				
HESI neg	No ions detected that correspond to the major ion in positive ion mode (at m/z 663)						

HESI heated electrospray ionization; APCI: atmospheric pressure chemical ionization; pos: positive; neg: negative

7.4. Discussion

Current anti-parasitic treatment for CD relies on the drugs benznidazole and nifurtimox, both associated with severe side effects and debatable efficacy in the chronic phase, which highlights the need to find novel anti-trypanosomal therapies (Bermudez et al. 2016; Zingales 2017). Recent efforts include improvement of current treatments, like combining benznidazole with other compounds or dosing adjustments, molecular targeted drug development, repositioning of known drugs, and discovery of novel compounds, like metal–drug complexes, chemically modified nitro-aromatic molecules, or plant-derived products (Bermudez et al. 2016; Moraes and Franco 2016). However, although many promising drugs have been

documented, others are needed due to the slow and rigorous validation process and high downstream failure of drug candidates (Bermudez et al. 2016; Moraes et al. 2014).

Plants represent an immense source of potentially bioactive molecules with anti-microbial activity including against *T. cruzi*, as for example rosemary (*Rosmarinus officinalis* L.) or green tea (*Camellia sinensis* (L.) Kuntze) (Bermudez et al. 2016), to name a few. But halophytes have been overlooked as prospective sources of anti-protozoal compounds, especially against *T. cruzi*. To the best of our knowledge, only Oliveira et al. (2016) screened several halophytes for *in vitro* anti-trypanosomal activity finding one extract from *Juncus acutus* L. roots able to decrease *T. cruzi*'s growth, while López et al. (2015) found that α-amyrine and quercetin isolated from the mangrove plant *Pelliciera rhizophorae* Planch. & Triana were active against *T. cruzi*. No reports were found in literature concerning the potential anti-parasitic activity of sea fennel and the everlasting towards *T. cruzi*, although aerial parts, including flowers, have reported anti-infective medicinal uses (Atia et al. 2011; Viegas et al. 2014). In this context, this work evaluated for the first time the *in vitro* anti-trypanosomal activity of decoctions, tinctures and essential oils from those aromatic halophytes against intracellular amastigotes of two *T. cruzi* strains. Anti-trypanosomal screenings are more relevant when performed against the intracellular amastigote since it better represents the *T. cruzi* tissue infection leading to CD and it is the main parasite form in the chronic stage (Zingales 2017; Palace-Berl et al. 2018).

Most of the tested samples did not yield promising anti-chagasic activity, either by low efficacy or due to high host cell toxicity, particularly when compared to the reference compound benznidazole (200 μM final concentration; Table 7.1). The exception was the decoction from sea fennel's flowers, which presented moderate activity with a percentage of infection reduction of 65% without affecting significantly the host cell. However, these results were obtained for the Y strain only, probably due to Sylvio X10/1 strain higher infectivity and superior number of intracellular amastigotes. The confirmatory dose-response screening of the active extract with the *T. cruzi* Y strain corroborated its anti-parasitic activity (EC_{50} = 17.7 μg/mL) and with no toxicity detected towards the host cells (Table 7.2, Figure 7.1). This active extract, sea fennel's flowers decoction, was characterized in terms of phenolic composition to identify individual phenolic compounds that could, in turn, be already described in literature as possessing anti-parasitic properties. The extract has mainly hydroxycinnamic acids, with chlorogenic acid as the dominant phenolic reaching almost 10 mg/g extract DW (Table 7.4). However, to the best of our knowledge, only Clavin et al. (2016) reported a weak *in vitro* activity of this phenolic against *T. cruzi* epimastigotes (IC_{50} > 100 μg/mL); Tasdemir et al.

(2008) describes its *in vitro* growth-inhibition against *T. brucei rhodesiense* amastigotes (IC_{50} = 18.9 μg/mL) but not against *T. cruzi*. Isomers neo and cryptochlorogenic acids have never been reported as anti-trypanosomal compounds. As for the remaining phenolics in sea fennel's flowers decoction, catechol and gallic acid revealed good *in vitro* trypanocidal activity against *T. cruzi* trypomastigotes (IC_{50} = 7.5 and 67 μg/mL, respectively; Tasdemir et al. 2006) and epicatechin had high *in vitro* lytic activity on bloodstream trypomastigotes (50% parasites lysed at 85 pM; Paveto et al. 2004), although not showing *in vitro* activity against *T. cruzi* trypomastigotes in another study (Tasdemir et al. 2006). Ferulic acid presented weak *in vitro* anti-*T. cruzi* trypomastigotes activity (IC_{50} > 150 μg/mL; Grecco et al. 2014), while coumaric acid displayed *in vitro* growth-inhibition of *T. b. rhodesiense* trypomastigotes but not of *T. cruzi* (Tasdemir et al. 2006); *p*-hydroxybenzoic and 4-hydroxybenzaldehyde were not found described as trypanocidal compounds. Overall, although some of the identified phenolics are portrayed as anti-trypanosomal, considering their reported IC_{50} and their lower amounts in the active extract (gallic acid, for example, was below the LOQ = 10 μg/g extract DW), only a synergistic effect between them could eventually justify the extract's anti-parasitic activity (EC_{50} = 17.7 μg/mL). Moreover, only Tasdemir et al. (2008) used *T. cruzi* amastigotes, the same life stage presently assayed, which can hamper this analysis of whether those phenolics are responsible for the extract's anti-protozoal activity considering that different *T. cruzi* forms can have different compound-susceptibilities. The amastigote form can be more difficult to affect since it is inside the host cells that have themselves protection mechanisms against cytotoxic agents (Izumi et al. 2011).

Sea fennel's flowers decoction was subsequently fractionated by liquid-liquid extraction and the resulting 5 fractions were assessed for anti-trypanosomal activity. The hexane fraction (fraction **1**) was the most active and selective and was further analysed and tentatively characterized by LC-HRMS. One major compound was present, likely the one responsible for the anti-trypanosomal activity, but the information provided by the HRMS analysis was, however, not enough for its identification (Table 7.5). Identification of compounds can be fairly easy when analytical standards are available but, in natural products research, analytical standards are often very expensive or not commercially available, leaving the identification task to be based on the available chromatographic and spectrometric information. In this case, that information was not sufficient to uncover the main compound in the active fraction. Nevertheless, a characterization by NMR (nuclear magnetic resonance) spectroscopy is currently underway and further research will confirm whether the major compound present is the active molecule.

Species belonging to the Apiaceae family, including sea fennel, have been shown to possess EOs with compounds active and selective against *T. brucei*, such as α-pinene, β-ocimene, limonene and sabinene, all monoterpenes found in *C. maritimum*'s EOs (Kamte et al. 2018). Another main compound already described in sea fennel's leaves, the polyacetylene falcarinol (also known as panaxynol; Cunsolo and Ruberto 1993), has been reported as very toxic (EC_{50} = 0.01 μg/mL) and highly selective against *T. b. brucei* (Herrmann et al. 2013). Moreover, a less abundant monoterpene found in sea fennel, linalool (Atia et al. 2011), showed potent trypanocidal effect against *T. cruzi* trypomastigotes (IC_{50} = 0.31 μg/mL; Villamizar et al. 2017). However, none of these compounds was identified as the main molecule in the active fraction 1. Nevertheless, monoterpenes and polyacetylenes represent classes of secondary metabolites with promising lead compounds to develop novel trypanocidal drugs (Kamte et al. 2018). To affect the intracellular amastigote form of the parasite, compounds must be able to pass through the host-cell's plasma membrane (Izumi et al. 2011). Several monoterpenes and polyacetylenes are lipophilic and can therefore cross the plasma membrane and disturb biomembranes within the cell (Wink 2008); monoterpenes in particular can cause destabilization of the protozoal plasma membrane and / or cause cell lysis (Raut and Karuppayil 2014). Overall, literature shows that there are many secondary plant metabolites that can have anti-trypanocidal activity and medicinal plants in particular, like sea fennel in the present study, can provide effective anti-parasitic molecules. The importance of identifying new trypanocidal compounds lies in the possibility of using them as novel or integrative therapies in CD treatment and / or as starting material for new drugs design.

6.5. Conclusions

To the best of our knowledge, this is the first report of sea fennel's *in vitro* anti-trypanosomal activity. Its flowers decoction showed activity against *T. cruzi* intracellular amastigotes with no toxicity towards the host cells; the anti-trypanosomal properties resided in fraction **1**. Despite some of the identified phenolics in the active extract being described as trypanocidal, the LC-HRMS tentative characterization of fraction **1** was not able to identify the major compound present, which is most likely responsible for the anti-trypanosomal activity. However, an NMR characterization of the fraction is currently underway. Additionally, when identified, structure–activity relationship (SAR) studies may disclose the mode of action and possible pharmacological applications of these molecules, encouraging further research.

Overall, this work shows that sea fennel can provide effective anti-parasitic molecule(s) with potential pharmacological application in the treatment of CD.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1. General Discussion

Traditional medicinal plants like, for example, the tea plant (*C. sinensis*), lemon verbena (*Aloysia citrodora* Palau), rooibos (*A. linearis*), chamomile (*Matricaria recutita* L.), coffee (*Coffea* species), and aloe (*Aloe vera* (L.) Burm.f.) are explored by the food industry as beverages or additives for functional foods (Gruenwald 2009; Pohl et al. 2016). Aloe, tea, chamomile, and other medicinal plants such as coconut (*Cocos nucifera* L.), ginkgo (*Ginkgo biloba* L.), ginseng (*Panax ginseng* C.A.Mey.), grape vine (*Vitis vinifera* L.), jojoba (*Simmondsia chinensis* (Link) C.K.Schneid.), lavender (*Lavandula angustifolia* Mill.), olive (*Olea europea* L.), sage (*Salvia officinalis* L.), turmeric (*Curcuma longa* L.), *Colchicum* and *Solanum* species, to name a few, are also ingredients in nutra- and pharmaceutical, cosmetic and nutricosmetic formulations for health purposes, beauty care and / or as overall well-being promoters (Aburjai and Natsheh 2003; Atanasov et al. 2015; Gruenwald 2009; Jivad and Rabiei 2014). However, medicinal halophytes are typically overlooked despite being great sources of highly bioactive molecules and of potential new-generation products with beneficial properties (Ksouri et al. 2012; Petropoulos et al. 2018a,b). In an effort to value medicinal halophytes, recent scientific endeavours have, nonetheless, set a new perspective on these salt-tolerant plants, namely their prospective uses as gourmet food (like *Sarcocornia perennis* (Mill.) A.J.Scott, *S. ramosissima*, and *A. macrostachyum*; Barreira et al. 2017), in herbal beverages (like *L. algarvense*; Rodrigues et al. 2016, 2017c), as sources of relevant molecules (e.g. juncunol, a potential anti-cancer drug from *J. acutus* L.; antiparasitic compounds from *J. acutus*, *I. crithmoides*, and *S. rubra*; Rodrigues et al. 2014; Oliveira et al. 2016, 2018), or as raw material for the cosmetic industry (like *P. lentiscus* and *A. pungens* as anti-hyperpigmentation; Lopes et al. 2016; Rodrigues et al. 2018b), the pharmaceutical industry (e.g.: *P. maritimum* and *L. algarvense* to manage diabetes and inflammation; Rodrigues et al. 2016, 2017b) and the nutraceutical / functional foods commercial segment (e.g.: *C. edulis*, *Juncus* species, *P. maritimum*, or *A. pungens*, as cognitive-enhancers or to mitigate age-associated conditions; Rocha et al. 2017; Rodrigues et al. 2017a, 2018a,b).

In this study, five medicinal halophyte plants common in the Algarve (southern Portugal), namely *A. campestris* subsp. *maritima* (dune wormwood), *C. maritimum* (sea fennel), *E. maritimum* (sea holly), *H. italicum* subsp. *picardii* (everlasting), and *P. coronopus* (buckshorn plantain), were selected for their traditional folk uses and promising biological activities, to unveil their potential as source of bioactive compounds and / or bioactive extracts with therapeutic, cosmetic and / or nutritional applications. Aiming at a consistent and timely

publication of the results, the work effort was focused at one halophyte at a time, rendering a chapter for each halophyte in the present thesis, plus a sixth chapter dedicated to the antiparasitic activity against *T. cruzi* (the causative agent of Chagas disease) of two aromatic species (sea fennel and everlasting). The present chapter endeavours a general discussion of the work developed, comparing the results obtained for each of the halophytes studied. It is important to mention that, while all plant extracts were to be sequentially extracted with solvents such as hexane, ethyl acetate, methanol and water, this was only accomplished with buckshorn plantain. For the other halophytes, preliminary results and contemporaneous literature being published dictated the need to alter this approach. Most literature studies deal with organic extracts despite the fact that traditional medicinal uses favour infusions, decoctions or tinctures of parts of, or the whole plant, to convey the plants' healing properties (Handa 2008). Hence, focusing on aqueous extracts seemed of greater significant importance to scientifically validate the folk-medicinal knowledge. Therefore, for the remaining four halophytes, extracts were prepared following the traditional uses of the plants, namely replicating infusions, decoctions and also tinctures in the case of the dune wormwood and sea holly. Moreover, for sea fennel, the everlasting and sea holly, tisanes (and tinctures for sea holly) were studied in their respective ingestion dosage, the "cup-of-tea" measure (or drops for sea holly's tinctures), to assess the plants from a medicinal use perspective, given that the "cup-of-tea" (200 mL) is a worldwide measure for drinking tisanes (for tinctures, 1 mL equals approximately 3 to 5 drops, which is a common ingestion therapeutic dosage).

8.1.1. Phytochemical profile

The chemical composition of halophytes comprised the analysis of known bioactive compounds such as phenolic compounds. As detailed in chapter 1, these are perhaps the most widely occurring group of secondary metabolites and some of the most important plant bioactive compounds, and herbal extracts are important sources of them (Balasundram et al. 2006). Hence, extracts from the five halophyte plants under study (different anatomical organs: roots, stems, leaves and / or flowers) were characterized in terms of phytochemicals present by spectrophotometric methods to determine total polyphenolic content (TPC) and other phenolic groups (total flavonoids, condensed tannins, etc). For simplification purposes, only the TPC will be presently discussed.

Although there is no established classification in terms of high / low values of total phenolics, some authors consider that natural extracts are rich in phenolic compounds if their

TPC (expressed as GAE, gallic acid equivalents) is higher than 20 mg/g DW (Kähkönen et al. 1999; Lopes et al. 2016; Rodrigues et al. 2015). This was the case for all organs of buckshorn plantain (roots 41.3, leaves 42.6, flowers 29.5 mg GAE/g DW) and of the dune wormwood (roots 114 – 118, aerial-organs 119 – 134 mg GAE/g DW), and also for all organs of sea fennel and the everlasting and most organs of sea holly (roots' tisanes and stems' tinctures were the exception) when considering the extraction yields obtained and the TPC per cup-of-tea (see Table 8.1).

Table 8.1 Yields (infusion and decoctions: g extract/200mL, tinctures: g extract/mL) and phenolic contents in infusions and decoctions (mg/cup-of-tea) from sea fennel, everlasting and sea holly, and tinctures (mg/mL) from sea holly.

Plant	Organ	Extract	Yields	TPC[a]
C. maritimum	Stems	Infusion	0.302	12.4 ± 0.36
(sea fennel)		Decoction	0.319	12.8 ± 0.92
	Leaves	Infusion	0.478	33.7 ± 0.91
		Decoction	0.500	35.3 ± 2.98
	Flowers	Infusion	0.404	21.2 ± 0.17
		Decoction	0.389	22.6 ± 0.99
H. italicum subsp. *picardii*	Roots	Infusion	0.071	13.9 ± 0.49
(everlasting)		Decoction	0.091	20.5 ± 1.45
	Vegetative aerial-organs	Infusion	0.239	62.0 ± 0.95
		Decoction	0.265	70.2 ± 4.35
	Flowers	Infusion	0.265	69.9 ± 3.88
		Decoction	0.326	76.5 ± 2.62
E. maritimum	Roots	Infusion	0.521	3.32 ± 0.29
(sea holly)		Decoction	0.443	3.72 ± 0.30
		Tincture	0.054	7.61 ± 0.65
	Stems	Infusion	0.214	4.25 ± 0.21
		Decoction	0.224	5.34 ± 0.40
		Tincture	0.023	0.42 ± 0.02
	Leaves	Infusion	0.350	12.0 ± 0.69
		Decoction	0.348	11.2 ± 0.82
		Tincture	0.028	0.88 ± 0.04
	Flowers	Infusion	0.263	17.0 ± 0.62
		Decoction	0.268	14.9 ± 0.39
		Tincture	0.025	0.76 ± 0.06

[a] TPC: total polyphenol content, mg GAE/200 mL or mg GAE/mL, GAE: gallic acid equivalents.

In this sense, these halophytes' extracts can be considered of high polyphenolic content and a potentially good source of these bioactive phytochemicals. Overall, in comparison with each other, the everlasting had the highest phenolic content, followed by the dune wormwood. Tisanes from sea holly showed the lowest phenolic contents, particularly when compared with herbal teas from sea fennel and everlasting. It is also noteworthy that, globally, aerial organs (leaves and flowers) from all studied plants had higher phenolic contents than the other organs, an accumulation pattern probably linked to the phenolics role 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 larger radiation-exposed areas, phago-deterrents against herbivores, or contribute to pigmentation that attracts pollinators (Harborne and Williams 2000; Hutzler et al. 1998; Petersen and Simmonds 2003).

Phenolic compounds in plant extracts can be extremely diverse, hence extracts were further characterized by High Performance Liquid Chromatography (HLPC) (in the case of buckshorn plantain, sea fennel and sea holly) or Ultra-HPLC – Photodiode Array – accurate mass-Mass Spectrometry (UHPLC-PDA-amMS) (in the case of the everlasting and dune wormwood) to determine their polyphenolic profile. The UHPLC-PDA-amMS also allowed the identification of other tentatively identifiable compounds in the more promising halophyte species (see section 8.1.4. Biological activities). A wide diversity of phenolics was identified in these halophyte plants, the most abundant ones being verbascoside and luteolin-7-*O*-glucoside in buckshorn plantain, chlorogenic, neo- and crypto-chlorogenic acids in sea fennel, quinic and chlorogenic acids in the everlasting and dune wormwood, and carvacrol and naringenin in sea holly. Phenolic acids were the most common phenolic compounds found among the studied plants, with a slightly higher prevalence of hydroxycinnamic acids (HCAs). HCAs occur in most plant tissues: they are incorporated into cell walls contributing to its mechanical strength, play a regulatory role in plant growth and morphogenesis, are involved in cells' responses to stress and pathogens, and often form the polymeric waxes coating plants' external surfaces (El-Seedi et al. 2012). The most common compounds determined were coumaric and ferulic acids, present in all five halophytes. These HCAs are associated with plant cell walls and have a role in plant growth, contributing to lignin biosynthesis, which may explain their occurrence in all of the studied plants (Lattanzio et al. 2006). Moreover, *p*-hydroxybenzoic, syringic and chlorogenic acids were present in at least four of the halophyte species. Some hydroxybenzoic acids, like *p*-hydroxybenzoic and syringic acids, seem to have allelopathic effects, inhibiting the nearby growth of others plant species (Seal et al. 2004; Weir

et al. 2004). Chlorogenic acid acts as an antioxidant, protecting plant tissues against oxidative stress (Niggeweg et al. 2004). It has also been associated with plant resistance to fungal attack (Friend 1979), and its synthesis is increased in response to plant wounding (Ananthakrishnan 1990). This may explain the occurrence of these three phenolic acids in most of the studied halophytes. Additionally, while the concentration of phenolic compounds varied considerably between the different halophytes' organs, overall, aerial organs (flowers and leaves) had the highest sum of identified compounds in all plants except sea holly (where roots had the higher quantity, mainly due to the dominant compound found in this organ, carvacrol). Again, the higher phenolic accumulation in aerial organs suggests their protective role in the above-ground organs, possibly against excessive UV-radiation, predation or towards reproductive success conferring appealing colours and / or taste to pollinators and / or seed dispersers.

These molecules determined in the plants' extracts have been shown to possess several biological activities with potential important health effects. To exemplify, the most abundant phenolics identified in the five halophyte plants are all strong antioxidant compounds (Gálvez et al. 2005; Patel et al. 2014; Pero et al. 2008; Santana-Gálvez et al. 2017; Shahidi and Ambigaipalan 2015), some with cytotoxic / anti-carcinogenic properties (carvacrol, chlorogenic acid, luteolin-7-*O*-glucoside, naringenin, verbascoside; Baser 2008, Gálvez et al. 2003; Meng et al. 2013; Patel et al. 2014) and antimicrobial activity (carvacrol, chlorogenic acid, verbascoside; Baser 2008; Egorov et al. 2004; Santana-Gálvez et al. 2017), others with anti-inflammatory capacity (naringenin and verbascoside; Alipieva et al. 2014; Patel et al. 2014) or even hypoglycaemic / anti-diabetic and hypo-lipidemic / anti-obesity properties (chlorogenic acid and naringenin; Meng et al. 2013; Nakajima et al. 2014; Patel et al. 2014). In fact, almost all the phenolics identified in the halophytes' extracts are described in literature as interesting and valuable natural bioactive phytochemicals, and their presence may help explain some of the plants' main traditional medicinal uses while also highlighting the potential use of these halophytes as a source of bioactive molecules and / or products with health promoting potential.

8.1.2. Mineral composition

Besides being important sources of polyphenolic compounds, herbal extracts may also be an added source of other components for the human diet such as minerals (Pohl et al. 2016). Additionally, halophytes have been referred as generally having high mineral content (Barreira et al. 2017; Buhmann and Papenbrock 2013; Guil-Guerrero 2001; Guil-Guerrero et al. 1998; Ventura et al. 2011), a trait potentially related to the saline environment in which they live (Díaz

et al. 2013; Redondo-Gómez et al. 2010). As previously mentioned, halophytes have unique features that allow them to cope with high salinity levels, including accumulation and compartmentalization of selective ions and compatible solutes for osmotic adjustment. Moreover, they can regulate transpiration and accumulate elements, as for example K, in the presence of high Na and Cl concentrations (Flowers et al. 2010). Therefore, the presence of minerals in the halophytes' extracts could be of added value for their potential use as food products. In this sense, the mineral content in the extracts of buckshorn plantain leaves and of sea fennel, dune wormwood and sea holly organs were analysed. Sea fennel and sea holly extracts were analysed considering the cup-of-tea (200 mL) measure. As expected, sodium was the most abundant element determined in the halophytes' extracts, particularly those from aerial-organs like leaves. Values of this element were higher than those usually found in regularly consumed tisanes from glycophyte plants like *M. recutita* (chamomile), *Cymbopogon citratus* (DC.) Stapf. (lemongrass), or *Tilia* species (linden) (Pohl et al. 2016), although not reaching the maximum recommended daily intake of Na (2000 mg; WHO 2012a) in terms of both cup-of-tea and gram of extract measures. The other macro-elements (Ca, K, Mg) were also fairly abundant in the halophytes' extracts, overall higher in leaves and flowers, supplying up to 2.0% of Ca (in sea fennel's leaves), 0.6% of K (in sea fennel and sea holly's flowers) and 2.9% of Mg (in sea fennel's leaves) of the adult daily recommended intakes (Ca: 1000 – 1300 mg/day, Mg: 190 – 260 mg/day, K: 3510 mg/day; WHO/FAO 2004; WHO 2012b). As for microelements (Fe, Mn, Zn), although found in lower concentrations, their presence in the extracts still represents up to 12% of Fe (in dune wormwood's roots), 1.8% Mn (in dune wormwood's aerial-organs) and 1.7% of Zn (in buckshorn plantain's leaves) of the recommended daily intakes (Fe: 9.1 – 58.5 mg/day, Zn: 3 – 14 mg/day, Mn: 5-5.5 mg/day; NHMRC 2006; WHO/FAO 2004; WHO 2012b). Tinctures from dune wormwood and sea holly had overall lower mineral content which was expected given the better ability of water to extract these nutrients from plants. Moreover, levels of potentially toxic minerals like Cu, Cr, Ni, Cd and Pb, when detected, were below legislated values for plants (Pb: 0.3 mg/kg wet weight, Cd: 0.2 mg/ kg wet weight of plant material, maximum levels in finished herbal products are not regulated; EC Regulation 1881/2006), pointing to the safe consumption of these extracts. Altogether, results highlight a possible nutritional role of these halophytes' herbal extracts as a mineral supplementary source, particularly from sea fennel for macronutrients and dune wormwood for microelements, therefore contributing to the adult daily intakes of some major and minor elements.

8.1.3. Toxicological assessment

It is crucial to determine the potential toxicity of new herbal products, such as plant extracts, to ascertain its safe consumption. Preliminary toxicological screenings of natural extracts can be assessed by *in vitro* models that address cytotoxicity of the extracts towards mammalian cell lines, delivering fast and reliable results and reducing *in vivo* testing (Nogueira et al. 2011; Rodrigues et al. 2016; Saad et al. 2006). As sea fennel, dune wormwood and everlasting extracts were, overall, the best promising extracts, they were submitted to a preliminary toxicological evaluation on N9 (murine microglia), S17 (murine bone marrow stromal), HepG2 (human hepatocellular carcinoma), and SH-SY5Y (human neuroblastoma) cell lines to assess cellular viabilities and therefore predict the extracts' potential toxicity. All samples had overall low toxicity. Sea fennel's samples in particular did not exhibited toxicity against both tumoural (SH-SY5Y, HepG2) and non-tumoural (N9, S17) cells, since cellular viability values were all equal to or higher than 90%. The everlasting's and dune wormwood's aqueous extracts had moderate to very low toxicity towards N9 cells (from 63 to > 90% cell viability values); S17 cells were more sensitive to toxic effects but, nevertheless, these halophytes' extracts had also moderate to low toxicity (53 – 77% viability values). Additionally, they exerted no toxic effects in the HepG2 cell line. However, sea wormwood's tinctures induced lower cellular viabilities for all cell lines than those obtained for the aqueous extracts. Green tea (*C. sinensis*) and rooibos (*A. linearis*) aqueous extracts were assessed alongside for comparison purposes as they are widely consumed as tea / tisanes. Cellular viabilities for these commercial plants' extracts were similar or even lower than those of the halophytes under study. Rooibos samples had moderate toxicity towards non-tumoural cells (56 – 66% viability values), as had green tea extracts against S17 cells (59 – 60% viability values). As a preliminary toxicological evaluation of these halophytes' extracts, results are quite promising particularly when compared to those obtained for the largely consumed green tea and rooibos tisanes, suggesting that they may be regarded as safe for consumption, specially the sea fennel. However, some caution is advised regarding the use of hydro-alcoholic extracts. *In vitro* toxicity studies of extracts from these plants are scarce but their large use for medicinal, nutritional and / or culinary purposes also points to their safe consumption. Nonetheless, although *in vitro* cell culture methods are accepted as an effective method for safety testing (Saad et al. 2006), further experiments on mammalian animal models should be pursued.

8.1.4. Biological activities

The five halophytes under study were tested for biological activities aiming at unravelling their potential therapeutic and health-promoting properties, while also attempting to validate their uses in folk medicine. All halophytes were tested through a battery of *in vitro* assays targeting antioxidant (radical scavenging and metal-related activities), anti-diabetic (digestive-enzymes inhibitory activity) and anti-hyperpigmentation (tyrosinase inhibiting activity) properties. However, not all halophytes had positive outcomes for these bioactivities and therefore each chapter detailed the bioactivities uncovered for each of the halophyte species. Other activities included in the work program, namely neuroprotective, anti-tumoural, and anti-obesity, were also tested but with unsuccessful results; they were not included in this thesis. A comparative analysis of the properties found in these plants follows.

8.1.4.1. Antioxidant properties

Antioxidant phytochemicals are scavengers of free radicals or ROS / RNS, and deactivators of metal catalysts by chelation, among other activities, reducing oxidative stress and consequent potential cell damage. It is increasingly documented that antioxidants effectively fight and prevent oxidative damage and thus are able to reduce the risk of oxidative stress-related conditions like neurodegenerative and vascular diseases, carcinogenesis or inflammation (Lu and Yen 2015; Saeidnia and Abdollahi 2013; Shahidi and Ambigaipalan 2015; Sindhi et al. 2013). Antioxidants are also associated with the management of *diabetes mellitus* (Sindhi et al. 2013) and amelioration of skin ageing conditions (Ribeiro et al. 2015). These are thus an essential group of potentially therapeutic / cosmetic molecules, also used as food additives to prevent harmful modification of oxidation-sensitive foods, such as oils and fats (Ksouri et al. 2012). Moreover, natural antioxidants are currently in high demand in the market as consumers are aware of the potential benefits of natural products and are willing to spend more on nutrition, cosmetics and supplements (Gruenwald 2009; Sindhi et al. 2013). In this context, there is a growing economic and health safety interest on the identification of halophyte species with high antioxidant content aiming at its use as an alternative to synthetic antioxidants in the food, cosmetic and pharmaceutical industries (Ksouri et al. 2012; Sindhi et al. 2013).

To understand the antioxidant potential of the halophytes under study, extracts were tested for radical scavenging activity using complementary *in vitro* assays, attending at the multifaceted aspects of antioxidants and their reactivity. Several methods targeting radical

scavenging activity (RSA) and redox metals were used to determine the antioxidant activity of the different extracts, namely the antiradical activity against DPPH, ABTS, NO, $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals, along with ferric reducing antioxidant power (FRAP) and metal chelating activities on Cu^{2+} and Fe^{2+}. For the everlasting, sea fennel, and sea holly extracts, results were expressed as percentage of activity in a cup-of-tea (and as % activity in 10 mg/mL extracts, for sea holly's tinctures). In these cases, a classification to describe the antioxidant activity of the extracts as potent (> 50% activity), moderate (30 – 50% activity) and low (< 30% activity) can be useful for comparison purposes (Vinutha et al. 2007; Custódio et al. 2015). Following this classification, almost all of the everlasting extracts had potent (> 50%) antioxidant activity (roots were the exception, week against the $OH^{\bullet}$ radical and at chelating copper), although being week (< 30%) in iron chelation. Similarly, most of sea fennel's extracts had potent RSA (only decoctions were potent NO and $OH^{\bullet}$ scavengers) and iron reducing capacity (FRAP), while being low to moderate (30 – 50%) at metal chelating. As for sea holly, tinctures showed overall potent antioxidant capacity (except against the NO radical and at chelating iron), whereas leaves and flowers aqueous extracts varied between moderate and potent antioxidant activity; however, stems and leaves' decoctions were the only potent iron chelators. Aqueous extracts of the commercial green tea and rooibos plants were assessed alongside for comparison purposes since they are sought for their strong antioxidant properties. Overall, the antioxidant activity of most of the everlasting's extracts matched or even surpassed that of green tea and rooibos, and the antioxidant capacity of the sea fennel's leaves and flowers samples were at least as effective as those of rooibos. For the dune wormwood and buckshorn plantain, results were expressed as IC_{50} values (mg/mL; half maximal inhibitory concentration, ascertained for extracts with activities > 50% at 10 mg/mL). The dune wormwood extracts were all effective at radical scavenging and at reducing iron, having IC_{50} values lower than 0.5 mg/mL for all assays. In fact, all extracts were better at scavenging NO and $O_2^{\bullet-}$ than the standard antioxidant controls (ascorbic acid and catechin, respectively) and almost all were similar or better DPPH scavengers than the standard control BHT. However, the chelating properties of this halophyte' extracts were moderate for copper (IC_{50} values: 1.3 – 3.6 mg/mL) and low for iron (IC_{50} values: 6.3 – > 10 mg/mL). As for the buckshorn plantain, globally, extracts had moderate to high RSA, particularly the roots' extracts towards DPPH (IC_{50} values: 0.5 – 0.8 mg/mL) and ABTS (IC_{50} values: 0.6 – 2.4 mg/mL) radicals. However, these samples did not match the standard antioxidants used in comparison. Metal chelating capacities of this plantain's extracts were moderate to low (IC_{50} values: Cu^{2+} 3.0 – > 10 mg/mL, Fe^{2+} 1.1 – >10 mg/mL). Comparing the five halophytes in terms of antioxidant potency is difficult considering the difference in the

study approach for the everlasting, sea fennel, and sea holly, where results were expressed as activity in a cup-of-tea. Still, the everlasting and dune wormwood seem to have scored potent antioxidant capacity in all of the assays, closely followed by sea fennel, while sea holly had the only potent iron chelating extracts; buckshorn plantain did not distinguish itself comparatively.

Several studies credit the pronounced antioxidant activity of halophytes' extracts to their polyphenolic content, an association not always verified in this work. Colorimetric assays give a TPC estimate and may not incorporate all the antioxidants in the extract (Tawaha et al. 2007), but the presence and amount of individual phenolics and possible additive / synergistic effects of constituents might help explain the discrepancy. Nevertheless, the phenolics' role as antioxidants, especially in halophyte plants, is widely reported (Ksouri et al. 2012). In summary, and bearing in mind other research studies on these five halophytes already detailed in the respective chapters, results confirm the strong *in vitro* antioxidant capacity of them and thus show these plants as useful sources of antioxidant compounds / products. Moreover, when considering the cup-of-tea / tisanes approach, those beverages may contribute to a regular dietary intake of oxidative-stress preventing molecules.

8.1.4.2. Anti-diabetic properties

Type 2 *diabetes mellitus* (T2DM) is a common metabolic disorder characterised by high plasma glucose levels (hyperglycaemia). It is the most common form of diabetes and affects millions of people, being estimated that about 450 million people will be affected by 2030. In modern medicine, the therapeutic strategy for T2DM management includes the inhibition of carbohydrate-hydrolysing enzymes, such as α-glucosidase and α-amylase, that results in the delay of carbohydrate digestion uptake, reduced postprandial blood glucose levels and therefore, in a reduction of hyperglycaemia linked to T2DM (Kumar et al. 2011; Patel et al. 2012). Although no reports were found on the use of the halophytes included in this work as sources of traditional anti-diabetic remedies, related species have long been used in traditional herbal medicine to manage T2DM (Hung et al. 2012; Patel et al. 2012), as for example *A. campestris* and *E. creticum* (Dib et al. 2016; Yaniv et al. 1987). In this context, the halophytes' extracts were assessed for their anti-diabetic potential by testing their capacity to inhibit α-glucosidase (microbial and mammalian) and α-amylase enzymes, in comparison with a known inhibitor clinically used, acarbose. From the five plants under study, extracts from the everlasting, dune wormwood and sea holly showed inhibitory activity: all three halophytes were active towards the microbial α-glucosidase, the dune wormwood and sea holly inhibited both microbial and mammalian α-glucosidase, but only sea holly was able to inhibit α-amylase.

Following the classification previously used to describe the potency of antioxidant extracts under the cup-of-tea measure, the everlasting's tisanes had a moderate to low inhibitory activity against microbial α-glucosidase (23 – 50%), as did those of sea holly (22 – 43%). Sea holly's tinctures (except from roots), on the other hand, can be considered as potent inhibitors (> 50%) of the three enzymes. Nevertheless, samples from these two species did not match the standard inhibitor used (acarbose at 10 mg/mL) in comparison for the three digestive-enzymes (82 – 96%). As for the dune wormwood, all of its extracts were more efficient at inhibiting the microbial α-glucosidase (IC_{50} values: 0.9 – 2.5 mg/mL) than acarbose, but only roots' extracts were able to inhibit mammalian α-glucosidase, particularly roots' tincture (IC_{50} = 2.90 mg/mL) that was more active than acarbose. Again, comparing these halophytes in terms of bioactivity potency is difficult considering the different approach between the everlasting and sea holly (activity in a cup-of-tea) and the dune wormwood (IC_{50} values). Still, sea holly was the only halophyte capable of inhibiting all three carbohydrate-hydrolysing enzymes while the dune wormwood scored higher efficiency at inhibiting α-glucosidase comparatively to the standard clinically used inhibitor acarbose.

It is recognized that polyphenolic phytochemicals, besides potent antioxidants (Ksouri et al. 2012; Shahidi and Ambigaipalan 2015), can modulate α-glucosidase and α-amylase activity therefore contributing to T2DM management by reducing post-prandial hyperglycaemia (Kumar et al. 2011; Hung et al. 2012). The stronger enzymatic inhibition of some extracts could be related to their constituents. For example, naringenin, catechin and carvacrol in sea holly's tinctures, chlorogenic acid and kaempferol 3-*O*-glucoside in the everlasting, and chlorogenic, caffeic and ferulic acids in the dune wormwood, are all phenolic compounds with some sort of anti-diabetic properties like hypoglycaemic and / or digestive-enzymes inhibitory activity (Alam et al. 2016; Govindaraju and Arulselvi 2018; Meena et al. 2017; Meng et al. 2013; Nakajima et al. 2014; Patel et al. 2014; Pereira et al. 2011; Sutherland et al. 2006). Overall, results suggest that extracts from these three halophytes, particularly those from the dune wormwood and sea holly's tinctures, could be beneficial in managing T2DM as they are capable of inhibiting dietary carbohydrate enzymes and consequently controlling glucose levels. Furthermore, oxidative stress has been implicated as a causative factor in the induction and progression of diabetes and diabetic complications by overproduction of ROS (Panigrahy et al. 2017; Saeidnia and Abdollahi 2013). In this sense, the extracts' combined antioxidant and anti-diabetic potential could help reduce oxidative stress and control glucose levels, benefiting T2DM patients and those at risk of developing the condition.

8.1.4.3. Anti-hyperpigmentation properties

Skin hyperpigmentation conditions like melasma, freckles, or age spots, are a result of melanin over-production. The enzyme tyrosinase is essential in melanin biosynthesis and therefore its inhibition can help prevent and / or manage undesired skin darkening (Khan 2007; Ribeiro et al. 2015). Tyrosinase is also responsible for unwanted browning of fruits and vegetables, which decreases their market value (Chang 2009; Khan 2007). Hence, tyrosinase inhibitors are increasingly sought not only for cosmetic purposes but also for their potential in improving food quality (Chang 2009; Khan 2007; Ribeiro et al. 2015). In this context, the anti-hyperpigmentation potential of the halophytes' extracts was evaluated by assessing their capacity to inhibit tyrosinase, in comparison with a known inhibitor (arbutin, a substance commonly used to treat hyperpigmentary disorders; Gillbro and Olsson 2011). However, from the five plants under study, only the extracts from the dune wormwood showed inhibitory activity. All its extracts were active, particularly aerial-organs' infusion (IC_{50} = 4.13 mg/mL), although less effective than the standard inhibitor arbutin (IC_{50} = 0.48 mg/mL). Some flavonoids were already identified as tyrosinase inhibitors, like quercetin, kaempferol and taxifolin (Chang 2009), all compounds detected in the dune wormwood's extracts that possibly contributed to their tyrosinase inhibitory activity. With natural products currently in high demand particularly for substances with beauty-enhancement properties like skin whitening or hyperpigmentation-preventing, this halophyte is a prospective source of molecules / products with applicability in preventing or managing undesired skin and food darkening.

8.1.4.4. Anti-protozoan properties

Little is known of halophytes potential use against neglected tropical diseases like Chagas disease (López et al. 2015; Oliveira et al. 2016), which is a potentially life-threatening zoonosis caused by the protozoan *T. cruzi*. The two aromatic halophytes, sea holly and the everlasting, have described anti-infective traditional uses and validated antimicrobial activities (Atia et al. 2011; Viegas et al. 2014), especially aerial organs, including flowers. Hence, they were selected to be tested for *in vitro* anti-trypanosomal activity by evaluating their natural extracts (decoctions, tinctures and essential oils from everlasting's flowers and sea fennel's stems, leaves and flowers) against intracellular amastigotes of two *T. cruzi* strains. From these two halophytes, sea fennel was active, namely its' flowers aqueous extract, with significant anti-trypanosomal activity (EC_{50} = 17.7 µg/mL) against one of the *T. cruzi* strains and no toxicity towards the host cells. Major phenolic compounds identified in this extract were chlorogenic acid and its isomers neo- and crypto-chlorogenic acids, but that phenolic has reported weak *in*

vitro activity against *T. cruzi* (IC_{50} > 100 µg/mL; Clavin et al. 2016) and its isomers were not found described as anti-trypanosomal compounds. Further fractionation of the active extract afforded 5 fractions from which the hexane fraction was the most active and selective (EC_{50} = 0.47 µg/mL); the clinically used positive control benznidazole had comparatively lower potency (EC_{50} = 0.92 µg/mL), although similar or higher selectivity. Tentative characterization of the active fraction was without success in identifying the major compound present, likely responsible for the anti-trypanosomal activity. To the best of our knowledge, this is the first report of sea fennel's *in vitro* anti-trypanosomal activity, showing that this halophyte can provide effective anti-parasitic molecule(s) with potential pharmacological application in the treatment of Chagas disease.

8.2. Synopsis / Conclusions

In this work, five halophyte plants with known ethnopharmacological value in the Mediterranean region were studied to unveil their potential as sources of bioactive compounds and /or bioactive extracts with therapeutic, cosmetic and / or nutritional applications. The greater scope was to expand our limited knowledge on the potential therapeutic and health-promoting properties of medicinal halophytes species.

In summary:

- **Buckshorn plantain** (*P. coronopus*; Chapter 2), traditionally used as analgesic, anti-inflammatory, antipyretic, anticancer, emollient and to treat disorders of the respiratory system (Ksouri et al. 2012; Redzic 2006), showed a high polyphenolic content, phenolic diversity and good antioxidant activity. Moreover, leaves can be considered as relevant sources of minerals. Altogether, results emphasize its potential as a source of bioactive molecules useful as antioxidants.

- **Dune wormwood** (*A. campestris* subsp. *maritima*; Chapter 3) is traditionally used to treat gastric disorders, hypertension, rheumatics, as anthelmintic and abortifacient (Almargem 2018); the species, *A. campestris*, is also described as having anti-diabetic, anti-inflammatory and antipyretic uses (Dib et al. 2016). Results show that this halophyte's extracts are a source of polyphenolic and mineral constituents,

antioxidants and α-glucosidase and tyrosinase inhibitors, pointing to a potential role in preventing oxidative-stress related diseases and managing T2DM and skin-hyperpigmentation conditions.

- **Sea fennel** (*C. maritimum*; Chapters 4 and 7) is described in folk medicine as appetizer, tonic, purgative, carminative, anthelmintic, diuretic, to prevent scurvy, and to treat renal and urinary complaints, digestive disorders, colic and inflammation of the urinary tract and prostate (Atia et al. 2011; Cornara et al. 2009; Franke 1982). Extracts had a high polyphenolic content, a strong antioxidant potential, and an interesting mineral profile, thus, they could be a potential source of bioactive molecules and / or products for the food industry, as for example antioxidants and minerals. Additionally, this halophyte had significant anti-*T. cruzi* activity, showing it may provide a source of anti-parasitic molecule(s) in the treatment of Chagas disease.

- **Everlasting** (*H. italicum* subsp. *picardii*; Chapters 5 and 7) is associated in folk therapeutics to analgesic properties and used in dermatologic, respiratory and digestive disorders with inflammatory, allergic or infectious components (Facino et al. 1990; Viegas et al. 2014). *Helichrysum* plants are also used as diuretic and to treat urinary disorders, burns, venomous bites and hernias (Viegas et al. 2014). Extracts from this plant had a high and diverse polyphenolic content, with similar or even higher antioxidant potential than the commercial green and herbal red teas, showing moderate α-glucosidase inhibition, pointing to a potential role in preventing oxidative-stress related diseases and managing T2DM.

- **Sea holly** (*E. maritimum*; Chapter 6) is a folk remedy used as diuretic, kidney stone inhibitor, aphrodisiac, expectorant, anthelmintic, antitoxin against various infections, for oedema reabsorption and / or pain relief (Erdem et al. 2015; Isermann and Rooney 2014; Lisciani et al. 1984). Results highlight that this halophyte's extracts could be a source of phenolic and mineral constituents, antioxidants and inhibitors of dietary carbohydrate digestive enzymes, pointing to their prospective role in managing T2DM and help preventing oxidative stress-related diseases.

Comparatively, the everlasting had the highest total phenolic content, followed by the dune wormwood while sea holly had the lowest TPC. Sea fennel was the better mineral supplementary source of macro-elements while dune wormwood was best for micro-nutrients. The everlasting and dune wormwood scored the highest antioxidant capacity, closely followed by sea fennel, but sea holly had the only potent iron-chelating extracts. Sea holly was capable of inhibiting all three carbohydrate-hydrolysing enzymes, but the dune wormwood scored higher efficiency at α-glucosidase inhibition. Additionally, only the dune wormwood had tyrosinase inhibitory capacity while only sea fennel was active against *T. cruzi*. Overall, all halophytes can be sources of bioactive molecules that, by being potent antioxidants, can help prevent oxidative stress-related diseases. However, only the dune wormwood combines antioxidant, anti-diabetic and anti-hyperpigmentation potential. Still, sea fennel's prospective anti-*T. cruzi* molecule(s) is of great pharmacological importance.

After these considerations, the **biological questions** posed in the objectives (section 1.5. Scope and Main Objectives of this Study) can be answered:

- **Can the traditional use of halophytes in folk medicine be supported by scientific evidence?** In part, yes. For example, the species *A. campestris* and another *Eryngium* species, *E. creticum*, are used for anti-diabetic purposes; our extracts from the *A. campestris* subspecies, dune wormwood, and the *Eryngium* species studied, sea holly, proved to have anti-diabetic potential by inhibiting digestive-enzymes. Furthermore, the wide diversity of phenolic compounds found in the studied halophytes are known for a wide range of biological activities and that can help explain the main traditional uses given to them. For example, buckshorn plantain has anti-inflammatory, analgesic and anti-pyretic uses, and some of its main phenolics like verbascoside and salicylic acid are associated with those properties. Additionally, the strong antioxidant capacity that these halophytes display may act to prevent or reduce the severity of conditions / diseases in which oxidative stress is an underlying factor, such as neurodegeneration, inflammation, carcinogenesis, diabetes, cardiovascular disorders, etc. It is noteworthy, however, that molecules that could be responsible for the alleged medicinal properties may have not been present in the extracts due to the solvents chosen to prepare them. Moreover, it is difficult to test / prove many of the claimed folk therapeutics for lack of applicable assays or due to logistics and time constrains.

- **Do these halophytes and / or their extracts have potential application as therapeutic, health-promoting or food-additive commodities?** Yes. For all five halophytes under study, results summarized above emphasize their strong potential as new sources of health-promoting products for the food, cosmetic and / or pharmaceutical industries. The dune wormwood in particular could deliver raw material to explore as a source of antioxidant, anti-diabetes and anti-hyperpigmentation molecules and even minerals. Furthermore, the unconventional approach taken regarding the "cup-of-tea" measure showed the great potential of tisanes from sea fennel, the everlasting and sea holly to be further explored as potential health-promoting food products as, for example, innovative herbal beverages.

- **Can these plants be good candidates for identification and commercialization of bioactive compounds and / or nutraceuticals from natural sources?** Yes. It is acknowledged that natural products are in high demand for positive outcomes particularly regarding anti-ageing, improving cognitive functions, beauty-enhancement, or manage conditions such as diabetes and obesity. Considering the potential health benefits of medicinal plants, this work confirms that medicinal halophytes in particular can offer a wide range of bioactive components, such as polyphenols, and reveal new sources of compounds, products or raw material for different commercial segments. In fact, amidst the plant kingdom, halophytes are an unexplored source of a new generation of foods, drugs and /or health-promoting products awaiting imaginative exploration.

Overall and in conclusion, the outcome of this work shows that the five halophyte species studied are great prospective candidates to use as food (buckshorn plantain leaves, for example), in herbal beverages (e.g. sea fennel, sea holly and dune wormwood), as sources of relevant molecules (e.g.: anti-*T. cruzi* compounds in sea fennel), or as raw material for the cosmetic industry (like dune wormwood for anti-hyperpigmentation), the pharmaceutical industry (e.g.: the everlasting, dune wormwood and sea holly to manage diabetes) and the nutraceutical / functional foods commercial segment (all five halophytes as strong antioxidants to prevent oxidative stress-related conditions). Hopefully, this thesis will contribute to raise awareness of the importance of these renewable, useful and full of potential resources.

As future research, a number of ideas can be considered. This work can, in the near future, be used to promote halophyte species from the Algarve as food ingredients, for example, to use in beverages, salads and / or soups, or supplements for functional foods. Future studies may

also include combined extracts from the halophytes with best results aiming at the formulation of cosmetic / pharmaceutical ingredients with stronger bioactivities, as for example antioxidant, anti-diabetic or anti-hyperpigmentation. Additionally, it would be interesting to perform *in vivo* studies to test the properties found in these plants in animal models. Finally, the sustainable cultivation of selected species for commercial purposes, should be pursued. Halophyte crops can be irrigated with different salt-water resources like seawater, brackish water, or marine aquaculture effluents in aquaponics, and these approaches, if successful, could allow the use of uncultivable saline soils and also potentially integrate marine aquaculture systems to mitigate its wastes.

8.3. References

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If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

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