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Faculdade de Farmácia

STUDY OF HALOPHYTE PLANTS PRODUCED IN PORTUGAL

Fábio Alexandre Sousa Andrade

Dissertation supervised by Doctor Sheila Alves and co-supervised by Professor Doctor Maria Rosário Bronze

Master's Degree in Food Quality and Health

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ABSTRACT

Halophyte plants recently became an interesting food ingredient, as they are known to present a high content in minerals that can contribute to salty taste and may be used as an alternative to traditional salt, and they have in their composition phytochemicals with antihypertensive effect. The main objective of this work was to characterize four halophyte plants produced in Portugal: *Crithmum maritimum*, *Inula crithmoides*, *Sarcocornia fruticosa* and *Salicornia ramosissima*, this later one produced under different culture conditions, natural environment and hydroponic culture. Results show that halophyte plants are good sources of fiber, protein, and polyunsaturated fatty acids. *S. ramosissima* produced in a natural environment presented the highest content in salt (NaCl) (5.62 g/100g fw). Ethanolic extracts prepared from each plant showed that a higher total phenolic content (TPC) (1.02 mg GAE/g fw) and antioxidant activity measured by ORAC and HOSC methods (23.8 and 26.1 µmol TEAC/g fw, respectively) was also detected for the same *S. ramosissima*. In general, all the extracts from these halophyte plants showed a rich profile in phenolic acids and flavonoid compounds. For the ACE inhibitory assay, there were no significant differences between the IC_{50} values from *S. fruticosa* and *S. ramosissima* from both origins (natural and hydroponic environment). In order to study the effect of two drying processes (conventional drying at 70 ºC for 3 days and lyophilization), *S. ramosissima* was used. Lyophilized *S. ramosissima* showed the highest TPC value (9.74 mg GAE/g dw) and antioxidant activity for ORAC and HOSC (418.7 and 237.2 µmol TEAC/g dw, respectively) when compared to the dried plant (7.41 mg GAE/g dw, and 291.1 and 147.2 µmol TEAC/g dw, respectively). The extract from the lyophilized halophyte plant compared to the dried plant also showed significantly higher contents for phenolic compounds such as quercetin-rhamnosyl-hexoside (88.04 µg/g dw), quercetin-malonyhexoside (4281.0 µg/g dw) and 3,5-dicaffeoylquinic (480.6 µg/g dw). Volatile compounds such as (E)-3-hexen-1-ol, 1-hexanol, *p*-cymene, 1,8-cineole, *β*-thujone, heptanal, 1-octen-3-ol, and methylbutanoic acid, some of them with characteristic odours, were identified in fresh, dried, and lyophilized *S. ramosissima*. Dried *S. ramosissima* was added to sweet and salty popcorn, and ketchup. Samples had a good acceptance from a consumer panel. Results obtained in this work show that halophyte plants can be an interesting alternative ingredient and their use as substitutes of traditional table salt must be studied.

Keywords: halophyte plants, antioxidant, antihypertensive, ingredient

RESUMO

As plantas halófitas são plantas que possuem a capacidade de crescer em zonas caracterizadas por concentrações salinas extremas e que, por consequência, possuem na sua composição um elevado teor em sal na forma de cloreto de sódio (NaCl). Estão já identificadas cerca de 5000 a 6000 espécies de plantas halófitas e a utilização destas plantas como possíveis ingredientes em alimentos tem-se revelado recentemente como uma área de investigação importante. Sendo o consumo de sódio um dos principais fatores a contribuir para a elevada incidência de hipertensão no mundo, é urgente procurar alternativas ao seu consumo e estas plantas constituem uma fonte de outros minerais que têm sido estudados como possíveis alternativas ao consumo de sódio. O potássio tem demonstrado atividade antihipertensiva e outros minerais como o cálcio e o magnésio podem ser interessantes na problemática da hipertensão. Paralelamente, estas plantas podem integrar, na sua composição, fitoquímicos que promovam uma diminuição da pressão arterial, como os ácidos fenólicos e flavonoides, que têm sido referidos como sendo compostos com atividade antihipertensiva. Assim, as plantas halófitas surgem como uma possível alternativa à utilização do sal convencional com a vantagem de contribuírem para a perceção do sabor salgado devido à presença de sódio, mas também de outros minerais, e possuírem ainda na sua composição compostos que são reconhecidos como tendo propriedades hipotensoras. Outras atividades biológicas como propriedades antibacteriana, antifúngica, hepatoprotetora, antioxidante, antitumoral, anti-inflamatória, e entre outras, têm sido também referidas na bibliografia.

Esta dissertação teve como objetivo principal avaliar as potencialidades nutricionais e caracterizar quimicamente plantas halófitas produzidas em Portugal. A escolha das plantas a estudar teve como base uma recolha de informação junto de diferentes produtores a operar no território nacional. Assim, foram escolhidas quatro espécies: *Crithmum maritimum*, *Inula crithmoides*, *Salicornia ramosissima* (R1 e R2) e *Sarcocornia fruticosa*. No caso da *Salicornia ramosissima*, foram estudadas duas proveniências (designação "R1" e "R2") que se caracterizavam por serem produzidas num ambiente natural (R1) e em hidroponia (planta R2). As restantes espécies estudadas cresceram em ambiente hidropónico.

Numa primeira fase do trabalho foram determinados os parâmetros nutricionais, a composição mineral, o perfil fitoquímico e a atividade antioxidante e antihipertensiva das

plantas halófitas selecionadas. Os resultados obtidos mostram que as cinco plantas halófitas selecionadas apresentaram uma percentagem de humidade superior a 85%. Estas plantas mostraram ser uma fonte interessante de proteína (valores superiores a 2,5 g/100g, exceção para *a S. ramosissima* (R1)), fibra (*C. maritimum, I. crithmoides* e *S. fruticosa* com valores superiores a 3,0 g/100g) e ácidos gordos polinsaturados (percentagens superiores a 50% do conteúdo de ácidos gordos total). Relativamente à composição mineral, esta variou entre as plantas halófitas estudadas, sendo que foram detetados valores mais elevados de sódio (22,50 ± 2,93 g/kg) e magnésio (1,09 ± 0,15 g/kg) na *S. ramosissima* (R1). Maiores teores de cálcio foram determinados na *I. crithmoides* (0,82 ± 0,10 g/kg). Teores mais elevados de potássio (3,50 ± 0,74 g/kg) foram doseados na *S. fruticosa*. No que se refere à presença de fitoquímicos, a *S. ramosissima* (R1) apresentou o teor mais elevado de compostos fenólicos totais (1,02 ± 0,04 mg GAE/g). Ácidos fenólicos como 3- e 5-cafeoilquínico, ácido *p*-cumárico e respetivos derivados, e flavonoides como a quercetina e seus derivados foram identificados nas quatro espécies de plantas halófitas estudadas. No que se refere à atividade biológica, foram avaliadas a atividade antioxidante (por ORAC e HOSC) e antihipertensiva (por ensaio inibitório de ACE). A *S. ramosissima* (R1) apresentou os melhores resultados de atividade antioxidante para os testes de ORAC e HOSC (23,8 ± 3,1 e 26,1 ± 2,3 μmol TEAC/g, respetivamente). No que se refere à atividade antihipertensiva, as plantas halófitas *S. fruticosa* e *S. ramosissima* (R1 e R2) apresentaram IC_{50}s idênticos (93,0 ± 7,9, 95,6 ± 14,1 e 102,3 ± 14,4 mg/ml, respetivamente).

Numa segunda fase do trabalho, selecionou-se uma das plantas, *S. ramosissima* (R1), que foi submetida a dois processos de secagem (secagem em forno convencional a 70 ºC durante 3 dias e liofilização) e os resultados obtidos no que se refere à sua composição química foram comparados. A seleção da planta halófita teve como base o facto de apresentar teor mais elevado de TPC, atividade antioxidante bem como de atividade antihipertensiva. Os resultados mostraram que os extratos etanólicos obtidos a partir da planta liofilizada apresentavam teores estatisticamente superiores de compostos fenólicos, 9,74 ± 0,88 mg GAE/g vs. 7,41 ± 0,29 mg GAE/g doseados na amostra sujeita a secagem convencional. No que se refere à atividade antioxidante, os resultados mostraram a mesma tendência, 418,8 ± 54,0 vs. 291,1 ± 17,9 μmol TEAC/g valores obtidos para o método de ORAC e 237,2 ± 12.0 vs. 147,2 ± 12,4 μmol TEAC/g para o método HOSC, respetivamente para a planta liofilizada vs. planta sujeita a secagem

convencional. A atividade antihipertensiva no ensaio inibitório de ACE para a *S. ramosissima* seca e liofilizada foi estatisticamente idêntico (24,6 ± 1,7 e 18,9 ± 0,6 mg/g, respetivamente). Foram ainda comparados os teores em quercetina-ramnosil-hexósido, quercetina-malonil-hexósido e 3,5-dicafeoilquínico que se revelaram superiores nos extratos da planta halófita liofilizada (88,04 ± 2,59, 4281,0 ± 24,1 e 480,6 ± 15,6 µg/g, respetivamente, vs. 52,90 ± 1,34, 1578 ± 30 e 223,4 ± 9,3 µg/g, respetivamente, na planta seca).

No que se refere à composição volátil, os resultados mostram que a planta *S. ramosissima* fresca e liofilizada apresentam teores superiores, expressos em áreas relativas, para compostos que são descritos como tendo odores a fresco e verde, tais como (E)-3-hexen-1-ol, 1-hexanol, *p*-cimeno, 1,8-cineole, *β*-tujone, e outros. A análise da planta halófita seca a 70 ºC permite detetar, numa percentagem de área relativa superior, compostos que são descritos como tendo odores menos agradáveis tais como heptanal, 1-octen-3-ol e ácido metilbutanoico, apesar da percentagem de área relativa elevada de hexanal, composto descrito com odores que remetem para o verde e ervas.

Apesar do método de secagem por liofilização apresentar vantagens no que se refere às determinações efetuadas, foi selecionada a *S. ramosissima* seca a 70 ºC para se desenvolver produtos que foram sujeitos a avaliação sensorial por consumidores. A escolha foi feita com base na facilidade de implementação a nível industrial do processo de secagem convencional em comparação com a liofilização, tendo em conta que este último processo apresenta custos mais elevados e é de maior complexidade.

Numa fase final do trabalho, a planta halófita *S. ramosissima* fresca e seca foi utilizada, em primeiro lugar, como ingrediente na preparação de pipocas doces e salgadas. Foram preparadas duas amostras distintas, onde a *S. ramosissima* fresca e seca foram usadas como substituintes do sal convencional nas amostras. Foi pedido a um painel de consumidores (n = 31) que avaliasse as duas amostras em termos de sabor e aparência. Os resultados demonstraram que, tanto em termos de aparência como de sabor, os consumidores preferiram a amostra de pipocas doces e salgadas com incorporação de *S. ramosissima* seca. A cor verde intensa na amostra com *S. ramosissima* fresca não foi considerada agradável para grande parte dos consumidores. Este resultado foi confirmado num segundo teste realizado com um número superior de provadores (n = 219). Estes resultados serão de valorizar tendo em vista o desenvolvimento de novos produtos com plantas halófitas.

Foram ainda desenvolvidos dois molhos *ketchup* em que se procedeu à adição de *S. ramosissima* seca em duas percentagens diferentes (2,2 e 3,0%). Foi realizada uma prova sensorial em que os consumidores (n = 102) avaliaram as amostras em termos de aparência, aroma e sabor. Para todos os atributos, houve uma preferência para a amostra com menor percentagem de *S. ramosissima*.

Em suma, este trabalho permitiu caracterizar plantas halófitas produzidas em Portugal e proceder a uma avaliação preliminar da possibilidade da sua introdução como ingrediente em alternativa ao uso de sal convencional. Dados os resultados positivos obtidos na avaliação sensorial, será importante no futuro perceber realmente a eficácia das halófitas como ingrediente funcional como uma estratégia de diminuir a incidência de hipertensão na população.

Palavras-chave: plantas halófitas, antioxidante, antihipertensiva, ingrediente

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ANOVA	One-way analysis of variance
DAD	Diode array detector
dw	Dried weight
ESI	Electrospray ionization
fw	Fresh weight
GAE	Gallic acid equivalent
GC	Gas chromatography
HOSC	Hydroxyl radical scavenging capacity
IC_{50}	Half maximal inhibitory concentration
LC	Liquid chromatography
LRI	Linear retention index
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
nd	not detected
ni	not identified
ORAC	Oxygen radical absorbance capacity
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
SPME	Solid-phase microextraction
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content

1) INTRODUCTION

1.1) Halophyte plants

Plants are at the base of medicine and, worldwide, their compounds are the main source for the production of drugs. Currently, the interest also falls on its possible introduction into food and, therefore, the study of plants in relation to their phytochemical composition and their possible different uses becomes important to explore [1].

About 70% of the Earth is covered by water and, considering environmental changes, such as global warming and freshwater reduction, more than 800 million hectares of land worldwide are affected by salinity [2–4]. This salinity, mostly on the coast, has been growing, which is problematic considering most of the plants cannot withstand high salt concentrations and don't have the capacity to grow on a salt affected land [5]. However, there is a category of plants that, due to the presence of different mechanisms in their constitution, has the ability to grow in extreme salt conditions, known as salt resisting or tolerating plants, or halophyte plants [2,5], which makes them good candidates to be cultivated in such soils [3].

Coastal regions, despite having a high NaCl content in the soil, provide an optimal habitat for halophyte plants [2]. These plants grow in areas such as salt marshes, where the coast is protected from the direct action of waves and currents, making the plants not being permanently submerged, but where there is influence of fresh water, sediment deposition, and smooth slopes [6]. Halophyte plants can withstand concentrations of salt higher than sea waters, being able to survive and thrive in soils with NaCl concentrations greater than 200 mM [4,6]. This resistance and survival to damage caused by salt stress is achieved by increasing the concentration of potassium ions (K^+) in the cell and the decrease in cellular sodium (Na^+), thus maintaining a favorable K^+/Na^+ ratio [4]. The accumulation and sequestration of inorganic ions allow the adjustment of their internal osmotic balance to external salinity [5].

Halophyte plants, which only represent 2% of terrestrial plant species (estimated 5000 to 6000 species) [5,7], can be divided into three groups, based on their salt demand: obligate halophyte plants, which need salt for their development; facultative halophyte plants, who prefer salt for their development but can grow well without or with a low salt concentration; and habitat-indifferent halophyte plants, those that prefer to live in a salt free soil but still tolerate salt during their development [5,8]. Halophyte plants can also

be classified based on their morphology, where they can be divided into: excretives, in case they are capable of excreting the salt in excess and, in this case, salt crystals can be visible on the leaf surface; or succulents, if they contain a salt bladder on their leaf surface, which causes them to store a large amount of water to minimize salt toxicity [5]. Furthermore, based on their habitat, halophyte plants can be called hydro-halophyte plants, if they grow in aquatic soil or in wet conditions; or xero-halophyte plants, which may grow with less water content [5].

1.1.1) Biological activities and main uses of halophyte plants

Plants are constantly studied due to the fact that they are excellent sources of secondary metabolites, such as alkaloids, terpenoids, tannins, and polyphenols. Given this, medicinal plants are expected to have a major importance on any pharmaceutical, cosmetic and even food industries [9]. In addition to the halophyte plants being consumed all over the world due to their organoleptic properties (taste, smell, and appearance) [10], their use in medicine has also been explored, due to their observed nutritional profiles and biological activities [6].

A large percentage of recent drugs are derived from plants or their products, and these secondary metabolites have shown numerous biological activities including antioxidant, antimicrobial, anti-depressant, anti-lipidemic, anti-inflammatory, antitumor, and others [9]. Halophyte plants have also been shown to be interesting in this respect, where biological activities, such as antifungal [11], antibacterial [12], hepatoprotective [13], antioxidant [14–16], antitumoral [17], anti-inflammatory [18], antihypertensive [19], and many others [20], have been reported.

Halophyte plants, in addition to being very promising for the use in areas affected by salinity and the creation of new green areas [21], have also been explored as promising ingredients to be used in a wide variety of foods [20]. Due to their distinctive chemical composition, their ability to withstand salt stress, their biological activities, and their functional characteristics, halophyte plants are recognized as very promising candidates for the food industry through their applicability as sources of natural ingredients for the development of new products with functional and beneficial health properties, such as beverages, salads, microencapsulated oils, food additives, antimicrobial agents, and others [20,22–24]. Despite the number of known halophyte plants species in the world,

their interesting use in the food industry, and being great sources of highly bioactive compounds, these plants are typically overlooked [25,26].

1.1.2) Halophyte plants as salt alternatives

Sodium chloride (NaCl), also known commonly as just salt, has been the focus of many studies in an attempt to replace this same ionic compound with other healthier alternatives, due to its constant direct connection with high blood pressure [27,28]. Halophyte plants, due to their salt content and, consequently, salty taste, are very promising when it comes to their introduction in food, especially in powdered form, to replace the conventional salt use [28,29].

The presence of minerals other than sodium, and bioactive compounds make halophyte plants attractive alternatives to sodium chloride as a food additive, due to the growing organic and natural markets [29]. *S. ramosissima*, an halophyte plant, has even attracted attention due to the commercial sale as "green salt" in Portugal [30]. In addition, studies have tried to take advantage of the functional features of the halophyte plants and use them as ingredients in certain foods, such as cooked sausages [27], bread [28], crispy fish cubes [24], and cookies [1]. Of the mentioned studies, only those developed by Lopes (2017) and Dias (2018) were the ones that used halophyte plants to replace the table salt in food and study the consequent effects.

Halophyte plants become an interesting ingredient to use as alternatives to salt in the sense of trying to reduce or replace the consumption of it, since hypertension is a current problem constantly present in the world population and salt consumption is one the main responsibles for the its occurrence [28]. However, this replacement still needs to be studied properly when it comes to its effectiveness in lowering sodium consumption.

1.2) Halophyte plants produced in Portugal

The interest and consumption of halophyte plants in gourmet cuisine in Portugal has been increasing in recent years [30]. In Portuguese territory, in addition to halophyte plants that grow along the country's seacoast, national companies have been dedicated to the production and commercialization of these plants, such as RiaFresh (Faro, Portugal), Horta da Ria (Aveiro, Portugal), and Salina Greens (Setúbal, Portugal). These companies are dedicated to species like *Salicornia ramosissima*, *Sarcocornia fruticosa*, *Inula*

crithmoides, *Crithmum maritimum*, *Halimione portulacoides*, *Atriplex halimus*, *Mesembryanhtemum crystallinum*, and many others.

1.2.1) Halophyte plants used in this study

For this study, it was selected a total of five halophyte plants produced in Portugal, corresponding to four different species: *Crithmum maritimum*, *Inula crithmoides* and *Sarcocornia fruticosa* from RiaFresh, and *Salicornia ramosissima* from both RiaFresh and Horta da Ria (**Figure 1.1**). *S. ramosissima* of two different companies was selected in order to compare the differences felt in the plant's growth in different environments. These halophyte plants were selected according to some parameters and in order to have a wide range of plants in the study. So, *C. maritimum* and *I. crithmoides* were selected due to the fact that both are aromatic halophyte plants and, consequently, are more gourmet-oriented, and *S. fruticosa* and *S. ramosissima* were chosen due to their already known presence in the Portuguese market and consequent ease of purchase. In addition, *C. maritimum* and *I. crithmoides* are more commonly being studied in the literature, as opposed to *S. fruticosa* and *S. ramosissima*.

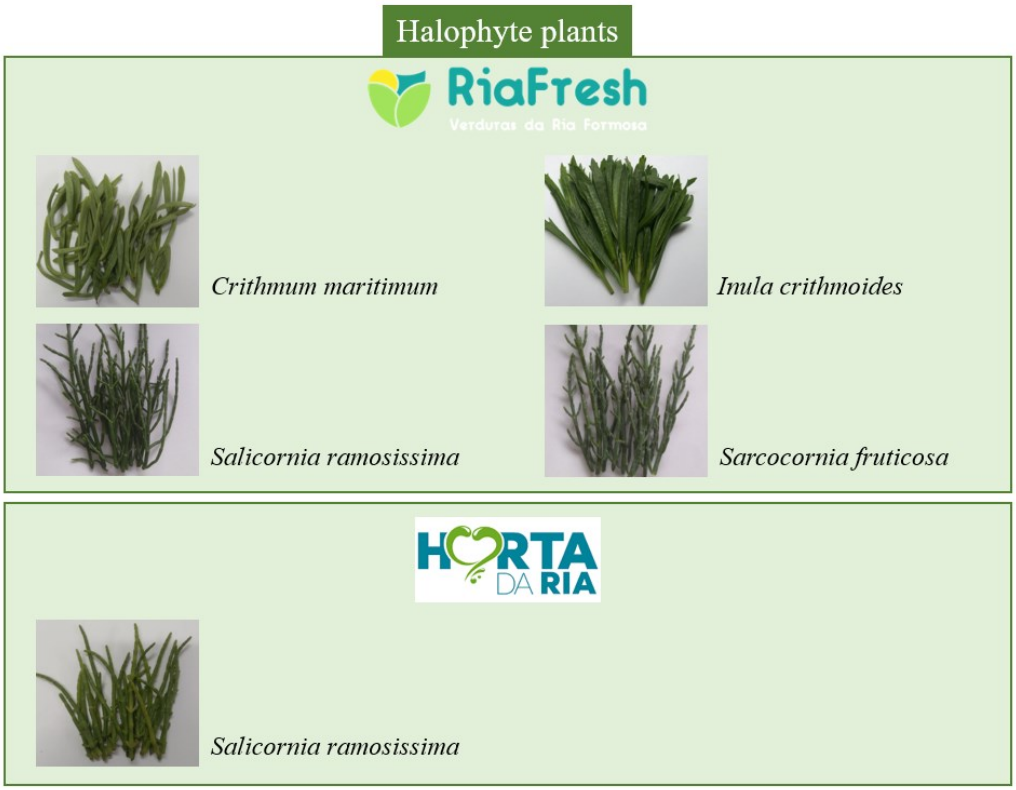

Figure 1.1 - Halophyte plants from RiaFresh and Horta da Ria studied in this work.

1.2.1.1) *Crithmum maritimum*

Crithmum maritimum is a facultative, perennial, and succulent halophyte plant from the Apiaceae family [15,20,31]. It is commonly known as sea or marine fennel, rock samphire, or crest marine, and usually grows on rocks, breakwaters, piers, and sandy beaches as those on the Atlantic coast of Portugal [15,32]. Traditionally, *C. maritimum* leaves were used for cooking, like in salads or as a condiment and pickle, due to its saltiness, and celery and citrus-like taste [33]. In addition, the plant was also used for medicinal purposes, due to its biological properties, such as antiscorbutic and diuretic activities [12,33]. The essential oil is used mainly in cosmetics [15].

Due to its phytochemical composition, *C. maritimum* has been extensively studied in the literature, when compared with the other plants present in this work. This plant is rich in vitamin C, iodine, carotenoids, omega-3 and omega-6 fatty acids, and phenolics compounds [12,15]. Volatile compounds such as *α*-terpinene, methyl carvacrol, *α*-pinene, *p*-cymene, sabinene, myrcene, *α*-thujene, camphene, *β*-thujene, *α*-terpinene, (Z)-*β*-ocimene, and terpinolene were reported in the literature [32,34]. Some phenolic compounds have been found in *C. maritimum*, such as caffeic, ferulic, and *p*-coumaric acids, caffeoylquinic acids and their derivatives, and quercetin and its derivatives [13,20,35]. *C. maritimum* has been studied due to its promising biological activities, such as antioxidant, antimicrobial, anti-inflammatory, and vasodilatory activities [12,15,20,36]. This halophyte plant becomes, then, very promising to be used in the pharmaceutical, cosmetic, and food industries.

1.2.1.2) *Inula crithmoides*

Inula crithmoides, also known as golden samphire, is a perennial and succulent halophyte plant that belongs to the Asteraceae family [12,20,37] and is commonly found in the Mediterranean basin and the Atlantic coast [38]. The aromatic young leaves of this halophyte plant are eaten raw or cooked, sometimes pickled, and used in salads [12]. In addition to being an edible halophyte plant, *I. crithmoides* was used traditionally for medicinal purposes, such as for treatment of bronchitis, tuberculosis, anemia, malaria, and diseases of urinary system [12,20].

I. crithmoides has been the focus of some studies, due to its chemical composition and variety of secondary metabolites. Compounds such as *α*-pinene, *p*-cymene, *β*-

phellandrene, and α-phellandrene were identified in the essential oil from the aerial parts of the halophyte plant [12,39]. In addition, caffeoylquinic acid and its derivatives have been identified in *I. crithmoides* [20]. Due to its antioxidant and antimicrobial activities [11,12,37], herbicidal potential [40], and sensory properties, this halophyte becomes interesting to be explored in the most diverse areas.

1.2.1.3) *Salicornia ramosissima*

The annual edible halophyte *Salicornia ramosissima*, also known as glasswort, sea asparagus, or green samphire, is part of the Amaranthaceae family [10,41,42]. This salt-tolerant succulent plant grows on the Portuguese Atlantic coast but also Mediterranean [3,10,43] and, due to its salty taste, it is widely appreciated in cuisine [10]. For this specific species, the traditional medicinal use is not known, but yet another species of the same *Salicornia* genus, *S. herbacea*, has shown its effectiveness against oxidative stress, inflammation, diabetes, asthma, hepatitis, cancer, and gastroenteritis [42].

This species does not have many studies in the literature, which makes it interesting to explore given its sensory characteristics and possible application as an ingredient in food [1,28]. However, the nutritional profile of *S. ramosissima* was recently studied [10] and phenolic compounds such as caffeic acid and quercetin were reported in significant quantities in a plant from the same genus [43]. Biological activities such as antioxidant, antihyperlipidemic, antidiabetic, antimicrobial, anti-inflammatory, and antiproliferative have also been reported in the *Salicornia* genus [44].

1.2.1.4) *Sarcocornia fruticosa*

Sarcocornia fruticosa from Amaranthaceae family is a succulent and salt-tolerant halophyte plant also found on the Portuguese Atlantic coast and Mediterranean basin [3,10,45]. *S. fruticosa* is distinguished from *S. ramosissima* due to the fact that it was identified as perennial [10,46]. This halophyte plant is also used in cuisine [10].

Like *S. ramosissima*, *S. fruticosa* does not have many studies in the literature, which makes its study and exploration more interesting, given its sensory characteristics. Recently, *S. fruticosa* demonstrated to be a good source of proteins, fibers, and minerals [38,47]. The plant also demonstrated antioxidant activity [38,48] and compounds like chlorogenic acid and its derivatives, and catechin derivatives were identified [47]. Other

halophyte plants from *Sarcocornia* genus were also studied nutritionally in the literature [10,45,46], which showed that these plants might be an alternative source of omega-3 polyunsaturated fatty acids for human consumption [46]. Phenolic compounds such as caffeic, ferulic, and *p*-coumaric acids, caffeoylquinic acids, and quercetin were identified in another plant of the *Sarcocornia* genus [45].

1.3) Hypertension

Blood pressure is the strength in which the blood circulates inside the vessels of our body. Hypertension occurs when this pressure is chronically higher than normal [49]. Therefore, hypertension is defined as a high or elevated pressure, and as a condition where the blood vessels have a constant increased pressure. The greater this pressure is, the greater difficulty the heart has during contraction [50]. A person is considered to have normal blood pressure values when both values are below 130/85 mmHg [49]. Blood pressure has two measurements: systolic blood pressure or "maximum" and diastolic blood pressure or "minimum". The first corresponds to the moment when the heart contracts, sending blood to the rest of the body, and the second corresponds to the moment when the heart relaxes to fill with blood again [50]. Values between 130-139 mmHg of systolic blood pressure and/or 85-89 mmHg of diastolic blood pressure are considered as normal-high values and, therefore, the person has a higher risk on developing hypertension. Someone is considered hypertensive if one of their blood pressure values, systolic or diastolic, or both, are equal to or greater than 140/90 mmHg, respectively [49].

The relation between blood pressure and cardiovascular and renal morbidity and mortality has been observed in certain studies, that did not show dependence with age and ethnicity [51–53]. Hypertension is one of the most, if not the most, important risk factor for countless heart diseases, such as coronary heart disease, hypertrophy of the left ventricle and valve heart diseases, cardiac arrhythmias including atrial fibrillation, stroke, and renal failure [51]. For the adult population, hypertension's definition is epidemiologic, so, the blood pressure of an adult might be considered abnormal when it is above a level which is associated with these several pathologies. In contrast, for kids and teenagers, the definition has a more statistical side, because there are no studies that determine what blood pressure levels are associated with possible future illnesses [50].

Among the important factors linked to elevated blood pressure, both genetic and environmental factors play the most important roles. Considering that hypertension involves these factors, it is commonly classified as a complex and multifactorial disease [50]. Most hypertensive patients present factors that can be modified, such as diabetes, overweight, sedentary lifestyle, smoking habits, salt ingestion, among others [49,54]. It is estimated that in Europe, 30-45% of the population has hypertension [49]. Due to the high incidence of hypertension in the general population, the modifiable factors become important to monitor and control, in the sense that by modifying them, it is possible to have a more controlled blood pressure [50].

1.3.1) Hypertension and salt

Over the years, the close and direct relationship between excessive salt consumption and hypertension, and excessive salt consumption and increased cardiovascular risk and mortality, has become increasingly approached and consolidated [55]. Although there are many salts, the most used in kitchen is sodium chloride (NaCl), which turns out to be only commonly known as "salt". Sodium is an indispensable cation for the well function of the body, essential in the action potential of all the cells of our body, and its homeostasis is under strong and controlled physiological regulation [55,56]. It is known that if sodium consumption is below the physiological range necessary for normal functioning of the body for long periods of time, deficiency conditions are likely to develop. However, the opposite is also true, where if sodium consumption is higher than the physiologically necessary level, a higher risk, adverse effects and even severe toxicity can also be observed [54,55].

One of the main things excessive sodium consumption causes is fluid retention, which leads to a high flow in the blood vessels, and it has been associated with the development of hypertension and the corresponding cardiovascular complications [55]. Blood pressure serves two purposes in our body: one is to maintain tissue perfusion, and the other extremely important function is the control of sodium balance, which largely determines the volume of extracellular fluid. Blood pressure is in fact the most important physiological mechanism in maintaining sodium and water balance [54]. In addition, there are other mechanisms that link excessive sodium consumption with increased blood pressure. An increase in salt intake leads to an increase in the generation of reactive oxygen species, which leads to an oxidative stress, and alterations in the extracellular

matrix of arterial wall, occurring an endothelial dysfunction [55]. Excessive salt consumption also leads to the activation of extracellular matrix metalloproteinases MMP2 and MMP9, leading to stimulation of TGFß-1, causing the breakdown of elastin and the accumulation of collagen [55]. This leads to an arterial stiffness, which causes an increase in systolic and diastolic pressures [55]. Furthermore, a high sodium intake stimulates aortic angiotensin II receptors, which stimulates the reception of angiotensin II, a vasoconstrictor peptide hormone [55,57]. This, then, raises the blood pressure by constricting the arteries [55,57]. **Figure 1.2** shows a summary of the mechanisms previously described.

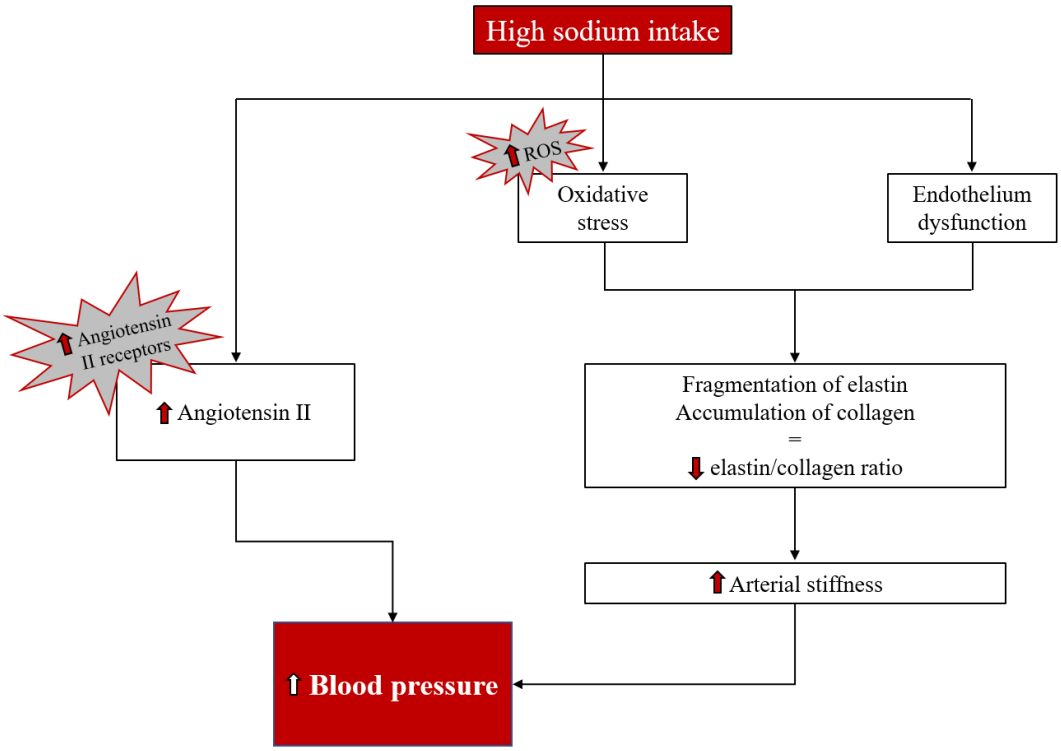

Figure 1.2 - Relationship between excessive sodium consumption and increased blood pressure. Adapted from Grillo et al. 2019 [55].

Reducing sodium intake has shown that it significantly reduces systolic and diastolic blood pressure in adults and children [58]. Studies show that reducing the sodium consumption not only reduces hypertension but also reduces the heart diseases morbidity and mortality [55]. Hypertension and the blood pressure increase due to age was shown to not be presented on populations where the individual consumption of sodium chloride is less than 50 mmol per day (equivalent to approximately 2.9 g of salt per day) [59]. The

World Health Organization (WHO) recommends reducing sodium consumption to less than 2 g per day (equivalent to 5 g of salt per day) with the aim of reducing blood pressure, and the risk of cardiovascular disease, stroke and heart disease in adults [58].

1.3.2) Use of salt alternatives to reduce blood pressure

Several studies have had their focus on searching for salt alternatives [60]. Some countries have given greater prominence to rich in potassium, calcium and magnesium, and low-sodium salts [54], and many industries have tried to develop other salt alternatives to the conventional salt [60]. There are two main categories of salt substitutes: rich in potassium salts, and herbs and spices. Potassium has a characteristic bitter taste and a lot of studies have been trying to find ways to reduce this bitterness [60]. On the other hand, using herbs, spices and other flavors can be safer, tastier, and healthier alternatives to conventional salt. Although herbs and spices can replace salt in the diet, they do not provide the salty taste and, therefore, it has become necessary to develop healthier salt substitutes that provide the missing salty taste [60].

When comparing developed populations with underdeveloped ones, it is observed a difference in the hypertension incidence in the population. This is mainly due to the difference in diets, where a higher consumption of processed foods in developed countries is associated with an incidence of hypertension in approximately one third of the population, due to their high sodium and low potassium content, while in the least developed countries, where the consumption of fruits and vegetables based foods is abundant, which are high in potassium and low in sodium, an incidence of less than 1% is observed [59].

An increase in potassium consumption has been shown to lower blood pressure values in hypertensive patients [54]. In addition, the sodium excretion, when in excess, is notably improved when there is an increase in the consumption of other minerals, such as potassium, magnesium and calcium [54]. Several mechanisms relate the potassium, calcium, and magnesium supplementation with the reduction of hypertension, such as influence on sympathetic nervous system activity, vasodilatory effects, and reduction of vasoconstrictor activities [54]. Despite the increase in consumption of potassium-based salts being able to protect against stroke, increase in blood pressure, heart rate problems, kidney failure and even osteoporosis, it may have undesirable effects on people with

difficulty in excreting excess or those who are already on antihypertensive medication that may function to increase the plasma potassium levels [60].

Due to the halophyte plants content and sensory characteristics, these type of plants are also an interesting alternative to table salt to be explored. Despite having a high sodium content in their constitution, halophyte plants have demonstrated their effect on reducing blood pressure, mainly due to the rich presence of phenolic compounds [20,61].

1.3.3) Phenolic compounds and their antihypertensive activity

Beyond primary metabolites, such as sugars, fatty acids, and amino and nucleic acids, there are secondary metabolites, which are not directly essential for basic photosynthetic or respiratory metabolism of plants, but are required for plants defense, protection, and survival [62]. In addition, secondary metabolites are structurally and chemically much more diverse than the primary metabolites [62]. Phenolic compounds are the most widely distributed secondary metabolites and have an aromatic ring with one or more hydroxyl groups [62,63]. Phenolic compounds such as phenolic acids, flavonoids, coumarins, tannins, lignans, and lignins are commonly found in plants [62,63]. The interest in phenolic compounds in particular comes from the several biological activities and health effects already known and reported in the literature, such as antioxidant, anti-inflammatory, antimicrobial, anticancer, and many others [62–64].

Within the numerous biological activities demonstrated by phenolic compounds, the antihypertensive activity is also mentioned. Phenolic compounds are interesting to be explored as natural antihypertensive drugs with the aim of replacing synthetic antihypertensive drugs, that sometimes cause severe side effects, despite their effectiveness [65,66].

A study demonstrated that the presence of rosmarinic and caffeic acids on a plant may be apparently linked to the antihypertensive activity demonstrated [67] and compounds such as ferulic, *p*-coumaric, caffeic, and ellagic acids showed a good correlation ($r > 0.90$) with the *in vitro* antihypertensive activity assessed [68]. Furthermore, numerous flavonoids and phenolic acids were identified in *Cuphea* species, namely miquelianin and quercetin, myricetin, and kaempferol derivatives, and a high inhibitory effect towards angiotensin-converting enzyme (ACE), enzyme that converts angiotensin I to vasoconstrictor angiotensin II (**Figure 1.3**), was observed [65]. Phenolic compounds such as chlorogenic

acid, isoquercitrin, catechin, epicatechin, vitexin, isovitexin, and procyanidins showed ACE inhibitory activity [69]. Another study carried out on an apple peel rich in flavonoids, such as quercetin derivatives, showed ACE inhibition efficacy, with quercetin 3-glucuronide showing the best antihypertensive activity [66]. Phenolic acids such as salicylic acid and caffeic acid were identified in the halophyte plant *Artemisia scoparia* and, when ACE inhibitory activity was tested, using quercetin as a positive control, caffeic acid showed a significantly higher antihypertensive activity than quercetin, and salicylic acid a slightly significantly lower activity than quercetin [61]. Flavonoids isorhamnetin 3-rutinoside, rutin, and quercetin were tested against ACE to evaluate their inhibitory activity, and quercetin was the one that demonstrated the greatest inhibition efficiency at the lowest concentration [70].

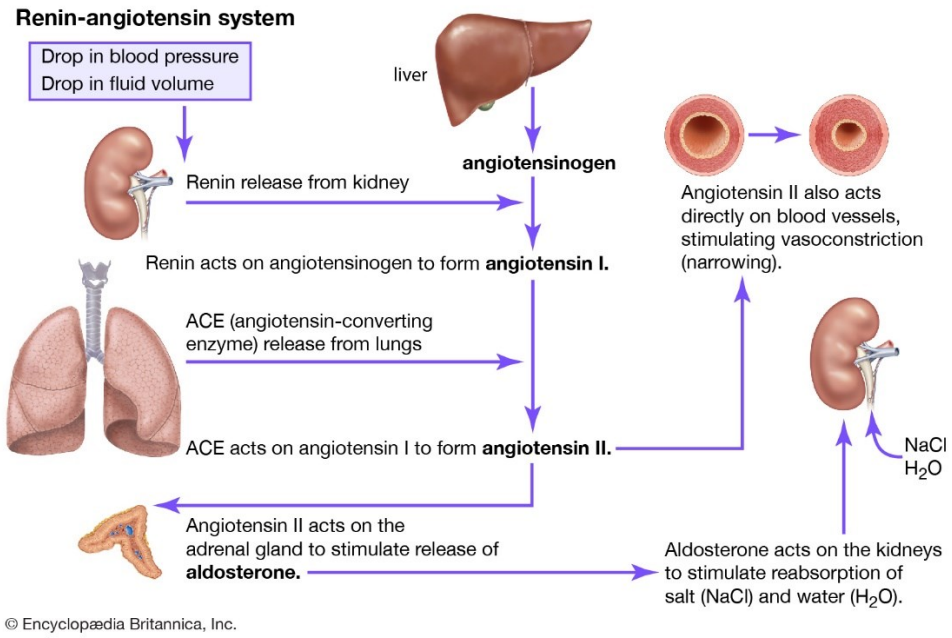

Figure 1.3 - Mechanism of the renin-angiotensin system [71].

Foods that are rich in phenolic compounds, especially some phenolic acids and flavonoids, become interesting to be explored due to their potential to be considered functional foods for the prevention and treatment of cardiovascular diseases.

1.4) Scope and main objectives of this study

The main objective of this study is to evaluate the advantages of using halophyte plants as ingredients in the preparation of foods. In order to accomplish this objective, the results

obtained in different tasks, described below, will be considered: (i) evaluation of the nutritional value and phytochemical composition, as well as the evaluation of the bioactivity, of halophyte plants produced in Portugal (*Crithmum maritimum* L., *Inula crithmoides* L., *Salicornia ramosissima* J.Woods and *Sarcocornia fruticosa* L. A. J. Scott); (ii) the effect of the drying process in the parameters previously described is also under study in one selected species, and (iii) the study of the effect of different growth environment (hydroponics vs wild) using halophyte plant *S. ramosissima* from two different regions of Portugal (Aveiro and Faro). Finally, the preparation of a product using these type of plants as ingredients will be evaluated using a consumer panel performing sensory acceptance tests.

2) MATERIALS AND METHODS

2.1) Plant material and processing

The halophyte plants were provided by two different companies dedicated to the production and commercialization of halophyte plants in Portugal, and, consequently, two different regions: Aveiro, Portugal and Faro, Portugal. The halophyte plants studied in this work as well as their relevant information are shown in **Table 2.1**. *S. ramosissima* was acquired from both regions (distinguished throughout the work by R1 and R2) possible comparison.

Table 2.1 - Inventory of the studied halophyte plants with origin, type of growth environment, harvest date, and parts of the plant used.

Scientific name	Origin	Growth environment	Harvest date	Part used
Crithmum maritimum L.	Faro, Portugal	Hydroponics	April/2019	Leaves
Inula crithmoides L.	Faro, Portugal	Hydroponics	January/2020	Leaves
Salicornia ramosissima J.Woods	Aveiro, Portugal (R1)	Wild	July/2019	All except root
	Faro, Portugal (R2)	Hydroponics	August/2019	
Sarcocornia fruticosa L. A. J. Scott	Faro, Portugal	Hydroponics	January/2020	All except root

In Part 2 of Results and discussion, *S. ramosissima* (R1) was chosen to study the influence of the drying process on the phenolic profile and bioactivity. The halophyte plant was dried in two different methods: dried at 70 ºC for 3 days in a heating oven with circulating air until complete drying, and lyophilized (ScanVac, Coolsafe 95/55-80 freeze drier, Denmark), that involves freezing, and then sublimation and drying under vacuum.

All plant material was stored at -18 ºC until analysis.

2.2) Reagents

The formic acid (HCOOH, 98% p.a.) and the acetonitrile (CH_3CN, 99.9% LC–MS) used in LC-DAD-ESI-MS/MS and LC-DAD were purchased from Merck and Fisher Scientific, respectively. The ultra-pure water (18.2 MO.cm) used throughout the work was obtained from a Millipore-Direct Q3 UV system (Millipore, USA). The Folin-Ciocalteu's reagent, Trolox, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and Fluorescein sodium salt were all acquired from Sigma-Aldrich. The chromatographic standard gallic acid was obtained from Sigma-Aldrich and the chromatographic standards 3-caffeoylquinic acid, quercetin-3-hexoside, and quercetin-3-acetylhexoside were purchased from Extrasynthese.

2.3) Nutritional profile and mineral composition

The analysis of the nutritional profile and the mineral composition of the halophyte plants was requested to Mérieux NutriSciences (Vila Nova de Gaia, Portugal), an independent company responsible for providing services abroad.

Protein content was determined by Kjeldahl method (F = 6.25) and ashes were quantified by means of sample incineration in a muffle furnace [72]. Total lipids were determined by Soxhlet extraction method and moisture in an oven at 105 ± 1 °C [72]. Carbohydrates were calculated by difference using the equation [Carbohydrates = 100 − (Ashes + Moisture + Protein + Lipids)] and total energy value was estimated using Atwater factor [73]. Total dietary fiber was determined using the enzymatic gravimetric [72]. Mineral composition was effectuated by flame atomic absorption spectroscopy [72]. The fatty acids composition of the samples was determined by gas chromatography (GC) with flame ionization detector (FID). The fatty acids methyl esters (FAME) were identified by comparison of retention times with FAME standard mixture under the same conditions (FAME Mix C4-C24, Sulpeco, USA) and quantified using area normalization. All measurements were performed in triplicate for each plant and for each analysis. Heavy metals were quantified using total X-ray fluorescence spectroscopy (TXRF) element analysis [74] by collaborator group Marine and Environmental Science Centre (MARE; Lisbon, Portugal). The created ketchup samples for the sensory tests were also analyzed nutritionally and minerally by Mérieux NutriSciences.

2.4) Phenolic compounds

2.4.1) Extraction of phenolic compounds

The extraction of phenolic compounds from halophyte plants was done using an ultrasound extraction method with some modifications [75,76]. Briefly, the fresh halophyte plants were smashed with mortar and pestle, with the help of liquid nitrogen. In the case of the dried and lyophilized *S. ramosissima*, the samples were grounded in a cyclone mill (Retsh). The phenolic compounds extraction of the fresh plants was done with ethanol and with the proportion: 10 g of smashed plant were extracted with 100 mL of ethanol:water (80:20, v/v) solution at room temperature. For dried *S. ramosissima*, the phenolic compounds extraction was done, similarly, but 2 g of dried *S. ramosissima* were extracted with 100 mL of ethanol:water (80:20, v/v) solution at room temperature. After shaking in vortex for 10 s, all samples were placed immediately in an ultrasonic water bath (ArgoLab DU-100, China). Extractions were performed using sealed vialsat 40 kHz of ultrasound frequency and 220 W of ultrasonic power for 60 min at 25 ± 3 °C, adding ice when needed to maintain the temperature. The samples were then centrifuged at 6000 g for 15 min (Sorvall ST16 centrifuge – Thermo Scientific, Germany) and the supernatant removed. The supernatant was evaporated to dryness at ± 40 °C under reduced pressure (120 Bar) using a rotavapor (Büchi R-114, Switzerland). The dry residue was dissolved in 2 mL of ethanol:water (50:50, v/v) solution, with the help of a glass sphere and vortex, filtered through a 0.22 mm SFCA membrane (Branchia, Spain) and stored at -18 °C until analysis.

All samples were extracted in triplicate.

2.4.2) Total phenolic content quantification

The total phenolic content (TPC) of the phenolic extracts of the different halophyte plants were determined according to Folin-Ciocalteu's method [77,78] adapted to microplate. Briefly, 10 µL of the extract, 230 µL of milli-Q water and 15 µL of Folin-Ciocalteu's reagent were mixed at room temperature for 3 min. Then, 45 µL of sodium carbonate solution (solution 35%) was added and the microplate was left to rest for 1 h at room temperature, protected from light. The absorbance was read at 765 nm in a microplate reader (Epoch2, Biotek (Winooski, USA)), with Gen5 3.02 data analysis software spectrophotometer. A gallic acid calibration curve was made in each test to be used for

the TPC quantification and the results were expressed in terms of gallic acid equivalent (mg GAE/g).

All measurements were performed in triplicate for each sample analyzed.

2.4.3) Identification of phenolic compounds by liquid chromatography (LC) coupled to mass spectrometry (MS) using electrospray ionization mass spectrometer (ESI/MS) and a diode array detector (DAD)

The phenolic compounds present in the halophyte plants extracts were analyzed in a Waters Alliance 2695 (Waters, Ireland) equipped with a quaternary pump, solvent degasser, auto-sampler, and column oven, coupled to a diode array detector (DAD) Waters 2996 (Waters, Ireland). A precolumn (100RP-18, 5 µm) and reversed phase C18 column (LiCrospher 100 RP-18, 250 × 4 mm; 5 µm) in a thermostatic oven at 35 °C were used for separation. The mobile phase consisted of water:formic acid (99.5%:0.5%) as eluent A and acetonitrile:formic acid (99.5%:0.5%) as eluent B at a flow rate of 0.30 ml/min. All solvents were filtered through a 0.22 µm PVDF membrane (Millipore, USA) prior to analysis. For the analysis, the following gradient elution program was used: 0-10 min from 99 to 95% A; 10-30 min, from 95 to 82% A; 30-44 min, from 82 to 64% A; 44-64 min, at 64% A; 64-90min, from 64 to 10% A; 90-100 min, at 10% A; 100-101 min, from 10 to 95% A; 101-120 min, at 95% A; finally returning to the initial conditions. The injection volume was 20 µl. DAD was used to scan wavelength absorption from 200 to 650 nm. A triple quadrupole mass spectrometer Micro-Mass Quattro micro (Micromass, Waters) outfitted with electrospray ionization source (ESI) was used in tandem at temperature of 120 °C, capillary voltage of 2.5 kV and cone voltage of 30 kV. The compounds were ionized in negative mode and spectra were recorded in the range *m/z* 60-1500. Analytical conditions were optimized to maximize the precursor ion signal ($[M-H]^-$). High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra-high purity argon (Ar) was used as collision gas. MassLynx software version 4.1 was used for data acquisition, processing, and analysis.

2.4.4) Quantification of phenolic compounds by liquid chromatography (LC) with diode array detector (DAD)

The LC system used was a UHPLC Vanquish (Thermo Fisher Scientific, USA), which is equipped with an auto-sampler, pump, and Vanquish diode array detector (DAD). Chromatographic separation of compounds present in fresh, dried, and lyophilized *S. ramosissima* extracts was carried on a Luna C18 reversed phase (Luna 5 µm C18(2) 100 Å, 250 x 4 mm; Phenomenex) and a Manu-cart RP-18 pre-column in a thermostatic oven at 35 °C. DAD was programmed for a scanning between 192 and 798 nm at a speed of 1 Hz with a bandwidth of 5 nm. The detection was monitored using three individual channels, 280, 320 and 360 nm, at a speed of 10 Hz with a bandwidth of 11 nm. The injection volume applied was 20 µl. The auto sampler's temperature was set at 12 °C. The mobile phase consisted of acetonitrile-formic acid (95%:0.5%) (eluent A) and acetonitrile;water:formic acid (90%:9.5%:0.5%) (eluent B), at a flow rate of 0.30 ml/min and injection volume of 20 µl. All solvents were filtered through a 0.22 µm PVDF membrane (Millipore, USA) prior to analysis. The system was run with the following gradient program: 0-10 min from 99 to 95% A; 10-30 min, from 95 to 82% A; 30-44 min, from 82 to 64% A; 44-64 min, at 64% A; 64-90min, from 64 to 10% A; 90-100 min, at 10% A; 100-101 min, from 10 to 95% A; 101-120 min, at 95% A; finally returning to the initial conditions.

The quantification was performed in duplicate.

2.5) Volatile compounds

2.5.1) Identification of volatile compounds by solid-phase microextraction (SPME) and gas chromatography (GC) coupled with mass spectrometry (MS)

For the extraction of volatile compounds from *S. ramosissima* (R1), the fresh plant material was smashed with mortar and pestle, with the help of liquid nitrogen, and the dried at 70 ºC and lyophilized material were grounded in a cyclone mill (Retsh). Then, 1.5 g of fresh plant material or, in the case of the dried plant material, 0.5 g, were added into a sample vial. Analyses were carried out in a SPME-GC-MS-QP2010 Plus (Shimadzu) equipped with an AOC-5000 autosampler (Shimadzu) and a capillary column Sapiens – 5-MS (Teknokroma), 30 m, 0.25 mm (IS), 0.25 µm (film thickness) was used. The working conditions were: the injector temperature was 250 °C, the splitless injection

mode was used, and the detector temperature was 250 °C. High-purity helium (≥99.999%) was used as the carrier gas, column oven temperature was kept at 40 °C for 5 min, increased to 170 °C at a rate of 5 °C/min, then was increased to 230 °C at 30 °C/min and maintained for 4 min; and carrier gas (He) with a flow of 2.00 ml/min. In MS interface, the temperature was 250 °C and the ion source temperature was 250 °C. Mass spectra were acquired in electron ionization (EI) mode at 70 eV in a *m/z* range between 29 and 300 with a scan speed of 588 scans/sec.

The identification of volatile compounds present on the three samples was made using the mass spectra library (NIST 2005 mass spectra database, Boulder, CO). The confirmation of the identification by the used library was made by calculating the Linear Retention Index (LRI), with the help of a standard mixture of alkanes (C_7-C_{20}):

$$\text{LRI} = 100\,\frac{t_r(c) - t_r(n)}{t_r(n+1) - t_r(n)} + 100\,n$$

where t_r is the retention time, c is the compound in question and n is the number of n-alkane hydrocarbons used for the calculation [79,80].

2.6) Bioactivity assays

2.6.1) Antioxidant activity assays

2.6.1.1) Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was used to evaluate the antioxidant capacity of the plant samples towards peroxyl radicals [81,82]. The assay was carried out using a microplate fluorescent reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of fluorescein (3.0×10^{-4} mM) catalyzed by peroxyl radicals generated from AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride). Briefly, in a 96-well flat-bottom black microplate, 25 µL of extract and 150 µL were mixed and then incubated at 37ºC for 10 minutes. 25 µL of AAPH was then added and the plate was read.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for quantification and all data was expressed as micromoles of Trolox equivalents antioxidant capacity per gram of plant material (µmol TEAC/g). All measurements were performed in triplicate for each sample analyzed.

2.6.1.2) Hydroxyl radical scavenging capacity (HOSC) assay

The HOSC assay [82,83] was performed using the FL800 microplate fluorescence reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). This assay evaluates the hydroxyl radical scavenging capacity of a sample using fluorescein (9.96×10^{-8} M) as a probe and a classic Fenton reaction with $FeCl_3$ (3.42 mM) and H_2O_2 (0.20 M) as a source of hydroxyl radicals. Briefly, 170 µL of fluorescein, 30 µL of extract, 40 µL of H_2O_2 and 60 µL of $FeCl_3$ were mixed in a 96-well flat-bottom black microplate and the plate was read at 37ºC for 60 minutes.

Samples were analyzed in triplicates and results were expressed as Trolox equivalents antioxidant capacity per gram of plant material (µmol TEAC/g).

2.6.2) Antihypertensive activity assay

2.6.2.1) Angiotensin-converting enzyme inhibition fluorometric assay

The antihypertensive activity of the different halophyte plants extracts was evaluated using an angiotensin-converting enzyme (ACE) activity assay kit (Sigma-Aldrich, Missouri, EUA). This fluorometric assay was adapted to allow the evaluation of the inhibitory activity of the halophyte plants towards ACE. Briefly, 10 µL of plant extract and 40 µL of ACE was added to a 96-well flat-bottom black microplate and incubated at 37ºC for 5 minutes to allow contact between the enzyme and the inhibitor. Then, 50 µL of substrate were added and the fluorescence was immediately read in kinetic mode in 5 cycles for 5 minutes.

A standard curve was used to allow the quantification of the formed product in each well with inhibitor and then the percentage of inhibition was calculated. Lisinopril (Sigma-Aldrich) was also used as a positive control.

2.7) Sensory analysis

2.7.1) Popcorn test

2.7.1.1) Popcorn formula and preparation

It was planned to insert *S. ramosissima* in a snack usually consumed by the Portuguese population, such as popcorn, in order to assess consumer acceptance in terms of visual acceptance and flavour. Initially, two different samples of sweet and salty popcorn, with the replacement of table salt by the halophyte plant, were prepared: one using fresh *S. ramosissima* (R2) and the other using dried at 70ºC *S. ramosissima* (R1). Briefly, sunflower oil (6 g) and 100 g of popcorn were added to a pan, which was covered and waited until almost formation of popcorn, while shaking at the same time. In a different pan, muscovado sugar (60 g) was used to make caramel and, after caramelization, *S. ramosissima* was added. After the popcorn formation, the salted caramel was added to the popcorn. The amount of *S. ramosissima* added was equivalent to 2 g of salt, meaning, 133.34 g of fresh *S. ramosissima* (R2) (which has 1.50 g in 100 g of fresh plant) was added to one sample (sample F1, in **Figure 2.1**) and 5.43 g of dried *S. ramosissima* (R1) (which has 36.8 g in 100 g of dried plant) was added to the other (sample D1, in **Figure 2.1**).

There was also the opportunity to perform a sensory test on the "ITQB Open Day". Here, for reasons of safety and hygiene, a sensory test was carried out just to assess the visual acceptance by a varied audience. In addition to using the previously prepared samples, one more was created, where a higher amount of fresh *S. ramosissima* was added (200 g) (sample F2, in **Figure 2.1**) when compared to the sample with fresh *S. ramosissima* previously prepared. This aimed to create a greener and, consequently, more visually different popcorn, to be able to assess how much people are willing to consume a product with a different appearance than normal.

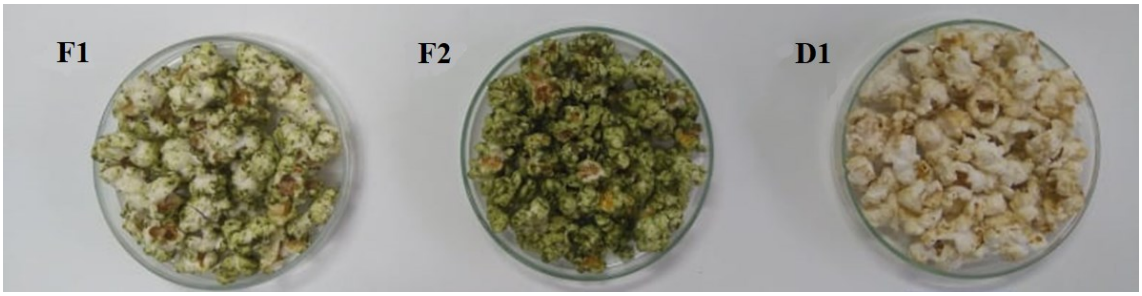

Figure 2.1 - Samples of sweet and salty popcorn prepared with 133.34 g (F1) and 200 g (F2) of fresh and dried (D1) *S. ramosissima*.

2.7.1.2) Test organization and testing

First, a test was performed with sweet and salty popcorn made with fresh (sample F1) and dried *S. ramosissima* (sample D1). People were asked first to put on the blindfold and taste each sample, with the assistant guidance. Each person rated the taste on a five-point hedonic scale: 1 = “disliked extremely”, 2 = “disliked”, 3 = “neither liked nor disliked”, 4 = “liked”, and 5 = “liked extremely”. Then, after being asked to remove the blindfold, they were asked to evaluate the appearance of each sample, also within the same five-point hedonic scale. To conclude the test, each person was also asked about their possible purchase intention in relation to each sample, choosing the option that best fit their intention ("wouldn’t buy", "would maybe buy", "would buy").

Considering the opportunity of having a sensory test on the "ITQB Open Day", participants were asked to evaluate the samples visually. In addition to the samples used in the previous test (samples F1 and D1), another sample with fresh *S. ramosissima* (sample F2) was prepared in order to assess the visual acceptance through a sample with a very prominent green color. Each participant was asked to evaluate appearance on the same five-point hedonic scale, as described previously, where 1 represented “disliked extremely” and 5 “liked extremely”. In addition, they were asked whether or not they would buy the samples evaluated, choosing the option that best suited their opinion ("wouldn’t buy", "would maybe buy", "would buy").

2.7.2) Ketchup test

2.7.2.1) Ketchup formula and preparation

Two ketchup formulations were prepared with dried *S. ramosissima* (R1) as a salt substitute, and fruit concentrate (natural sweeteners). The difference between the two ketchups was in the content of *S. ramosissima* added to the product: one had 2.2% (0.91 g of salt per 100 g of dried plant) of *S. ramosissima* (sample 2.2%DS, in **Figure 2.2**) and the other 3.0% (1.38 g of salt per 100 g of dried plant) (sample 3.0%DS, in **Figure 2.2**) (**Appendix A**). The samples were produced by Mendes Gonçalves (Golegã, Portugal) and they were obtained by homogenizing the tomato products with other natural ingredients, without the addition of refined sugar or salt.

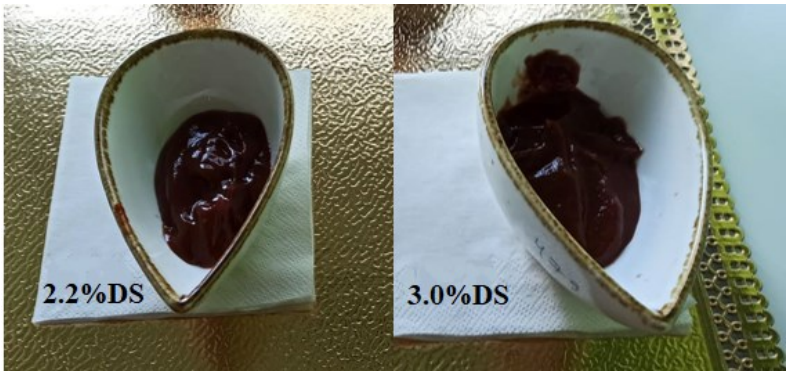

Figure 2.2 - Ketchup samples prepared with 2.2% (sample 2.2%DS) and 3.0% (sample 3.0%DS) of dried *S. ramosissima*.

2.7.2.2) Test organization and testing

A sensory test was organized with the two ketchups created with the aim to assess consumer's preference for a product containing different proportions of dried *S. ramosissima*, as well as the preference for a low in salt products. A questionnaire was carried out that accompanied the consumer throughout the test and where he would evaluate each of the ketchups. This questionnaire had some introductory questions, which allowed to know not only some information about the consumer, but also how each consumer was familiar with the product in their daily lives. After the introductory questions, the consumers were asked to evaluate the different attributes considered important of the samples, such as appearance, aroma, and taste. Consumers rated the sensory characteristics of the products on a nine-point hedonic scale: 1 = “disliked extremely”, 2 = “disliked very much”, 3 = “disliked moderately”, 4 = “disliked slightly”,

5 = “neither liked nor disliked”, 6 = “liked slightly”, 7 = “liked moderately”, 8 = “liked very much”, and 9 = “liked extremely”. Each consumer was also asked to evaluate, also on a nine-point scale, their overall impression for each of the samples. In addition, each consumer demonstrated its possible purchase intention for the preferred product, choosing the option that best fit their intention ("wouldn’t buy", "would maybe buy", "would buy"), and whether or not they would be willing to pay twice the price, compared to the price of a conventional ketchup found on the market, knowing that it would be a ketchup with the addition of an halophyte plant.

The tasting table consisted of well-identified individual containers for each sample, plastic stirrers to taste the ketchup and discard, trash can, napkins, water and glass, questionnaire, and pen. The test was carried out individually (one person at a time), allowing privacy for the consumer during the test.

2.8) Statistical analysis

The results obtained in the experimental part of this work were expressed as mean ± standard deviation and the statistical analysis was performed using the GraphPad Prism version 8.4.3 software (GraphPad Software, Inc., La Jolla, USA). For the antihypertensive assay, the IC_{50} values for each halophyte plant extract were calculated using the Non-linear regression (dose-response inhibition) in GraphPad Prism version 8.4.3 software. All the other results were submitted to one-way analysis of variance (ANOVA) followed by Tukey’s Test or unpaired t test, also using the GraphPad Prism version 8.4.3 software. The differences between the means at the 5% level ($p < 0.05$, meaning, within a 95% confidence interval) were considered significant and letters were used to establish these differences. Pearson correlations were also made in GraphPad Prism version 8.4.3 software. All graphics presented in this work were also designed using the GraphPad Prism version 8.4.3 software.

3) RESULTS AND DISCUSSION

Part 1: Characterization of different species of halophyte plants produced in Portugal - Nutritional value, phytochemical composition, and bioactivities

The first part of this work aimed to study a diverse group of halophyte plants for later selection and application as an ingredient in food. These plants were selected based on the collected information from different producers operating in the national territory, but also according to their sensory characteristics and studies previously reported in the literature, as previously described in the Introduction. In addition, the results of one species but from different regions and growth environments (hydroponics vs wild) were eventually compared.

3.1) Nutritional profile and mineral composition

The selected halophyte plants were nutritionally characterized in terms of water content, proteins, lipids, fibers, ashes, carbohydrates, energy, and salt (NaCl content). The nutritional parameters were expressed in g/100g of fresh weight (g/100g fw) and are shown in **Table 3.1**.

Table 3.1 – Nutritional composition and fatty acids profile of the halophyte plants.

	C. maritimum	***I. crithmoides***	***S. fruticosa***	***S. ramosissima***	
Location	Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
Nutritional composition (g/100g fw)					
Moisture (%)	88.80 ± 0.89^{b}	90.20 ± 0.90^{ab}	85.60 ± 0.87^{c}	88.20 ± 0.88^{b}	91.30 ± 0.91^{a}
Proteins	3.98 ± 0.15^{a}	3.29 ± 0.13^{b}	4.26 ± 0.14^{a}	1.59 ± 0.06^{d}	2.65 ± 0.10^{c}
Total fat	0.500 ± 0.005^{b}	0.500 ± 0.005^{b}	0.600 ± 0.006^{a}	0.400 ± 0.004^{c}	0.200 ± 0.002^{d}
Ashes	2.18 ± 0.09^{e}	2.73 ± 0.11^{d}	4.49 ± 0.18^{b}	5.91 ± 0.24^{a}	3.44 ± 0.14^{c}
Total dietary fiber	4.40 ± 0.13^{a}	3.10 ± 0.09^{c}	3.70 ± 0.11^{b}	1.00 ± 0.03^{e}	2.10 ± 0.06^{d}
Carbohydrates	0.14 ± 0.05^{d}	0.20 ± 0.008^{cd}	1.35 ± 0.05^{b}	2.90 ± 0.12^{a}	0.31 ± 0.01^{c}
Energy (kcal/100g fw)	29.20 ± 1.17^{b}	23.90 ± 0.96^{c}	35.0 ± 1.4^{a}	23.60 ± 0.94^{c}	16.60 ± 0.66^{d}
Salt	1.42 ± 0.18^{c}	1.35 ± 0.18^{c}	3.25 ± 0.42^{b}	5.62 ± 0.73^{a}	1.50 ± 0.19^{c}
Fatty acids profile (%)					
Palmitic acid (C16:0)	19.30 ± 0.01^{d}	18.60 ± 0.01^{e}	20.00 ± 0.01^{c}	24.00 ± 0.01^{a}	22.80 ± 0.01^{b}

	C. maritimum	*I. crithmoides*	*S. fruticosa*	*S. ramosissima*	
Location	Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
Stearic acid (C18:0)	3.80 ± 0.01^{a}	2.20 ± 0.01^{c}	1.60 ± 0.01^{e}	2.00 ± 0.01^{d}	3.10 ± 0.01^{b}
Oleic acid (C18:1)	7.10 ± 0.01^{a}	4.80 ± 0.01^{c}	5.70 ± 0.01^{b}	4.20 ± 0.01^{d}	4.80 ± 0.01^{c}
Linoleic acid (C18:2)	28.20 ± 0.01^{a}	23.50 ± 0.01^{c}	22.40 ± 0.01^{d}	24.00 ± 0.01^{b}	15.40 ± 0.01^{e}
Linolenic acid (C18:3)	23.50 ± 0.01^{e}	46.30 ± 0.01^{b}	45.10 ± 0.01^{c}	33.30 ± 0.01^{d}	46.50 ± 0.01^{a}
Arachidic acid (C20:0)	1.20 ± 0.01^{a}	0.50 ± 0.01^{c}	0.40 ± 0.01^{d}	0.90 ± 0.01^{b}	0.50 ± 0.01^{c}
Eicosenoic acid (C20:1)	0.20 ± 0.01^{b}	0.10 ± 0.01^{c}	0.40 ± 0.01^{a}	nd	0.10 ± 0.01^{c}
Behenic acid (C22:0)	1.20 ± 0.01^{b}	0.50 ± 0.01^{d}	0.70 ± 0.01^{c}	1.40 ± 0.01^{a}	0.70 ± 0.01^{c}
Lignoceric acid (C24:0)	1.90 ± 0.01^{a}	0.60 ± 0.01^{e}	1.00 ± 0.01^{d}	1.70 ± 0.01^{b}	1.60 ± 0.01^{c}
Σ SFA	30.20 ± 0.01^{c}	23.60 ± 0.01^{e}	24.40 ±0.01^{d}	34.30 ± 0.01^{a}	30.5 ± 0.01^{b}
Σ MUFA	17.30 ± 0.01^{a}	6.20 ± 0.01^{e}	6.50 ± 0.01^{c}	7.30 ± 0.01^{b}	6.40 ± 0.01^{d}
Σ PUFA	52.50 ± 0.01^{e}	70.20 ± 0.01^{a}	69.10 ± 0.01^{b}	58.40 ± 0.01^{d}	63.10 ± 0.01^{c}
PUFA/SFA	1.74 ± 0.01^{d}	2.97 ± 0.01^{a}	2.83 ± 0.01^{b}	1.70 ± 0.01^{e}	2.07 ± 0.01^{c}

nd – not detected (limit of quantification = 0.05 g/100g), SFA – total saturated fatty acids, MUFA – total monounsaturated fatty acids, PUFA – total polyunsaturated fatty acids. Results are presented as mean ± SD. In each row, the letters (a-e) mean statistically significant differences ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

The halophyte plants analyzed have a high percentage of water in their composition, with values higher than 85%: *S. fruticosa* having the significantly lowest percentage (85.60%), and *S. ramosissima* (R2) and *I. crithmoides* the significantly highest (91.30% and 90.20%, respectively). It is known that one of the main factors responsible for halophyte plants adaptation in extreme conditions is the hydration maintenance [84]. All the selected halophyte plants in this study are considered succulents [38,43,85], and the existence of succulent aerial parts contributes to the plant's protection [84]. The moisture values confirm this succulent designation.

Plant proteins are rich in non-essential amino acids, as opposed to the animal proteins which contain a greater content of essential amino acids, and they have shown to provide protective effects against cardiovascular diseases and cancer [86]. The highest protein

values were found in *S. fruticosa* and *C. maritimum* (4.26 and 3.98 g/100 fw, respectively), and *S. ramosissima* (R1) shows the significantly lowest value (1.59 g/100g fw). The high salt concentrations may lead to a low protein content due to possible toxic cytosolic concentrations of sodium that may hinder the protein synthesis [84]. This is in agreement with the values presented in this work, where *S. ramosissima* (R1) showed the highest salt content (5.62 g/100 fw) and the lowest protein content, and *C. maritimum*, which has one of the lowest salt content (1.42 g/100g fw), has one of the highest protein content. The ash content is highly correlated with the concentration in salt [84]. The significantly greater value of ashes in *S. ramosissima* (R1) (5.91 g/100g fw) is justified by the significantly higher detected salt content, but also other salts that may be present due to the mineral composition also analyzed later in this work. The opposite is also visible, where *C. maritimum* with the significantly lowest ash content (2.18 g/100g fw), also has one of the lowest content of salt in its constitution.

S. fruticosa demonstrated the highest total fat content and *S. ramosissima* (R2) the lowest (0.600 and 0.200 g/100g fw, respectively). Halophyte plants have been described as containing high dietary fiber contents [10]. Dietary fiber provides many health benefits, reducing the risk of developing diabetes, obesity, hypertension, and others [87]. *S. fruticosa* displays the highest value (3.70 g/100g fw) of dietary fiber content, while *S. ramosissima* (R1) the lowest (1.00 g/100g fw). *S. ramosissima* (R1), on the other hand, shows the significantly highest value of total carbohydrates (2.90 g/100g fw). These results are shown in **Figure 3.1**.

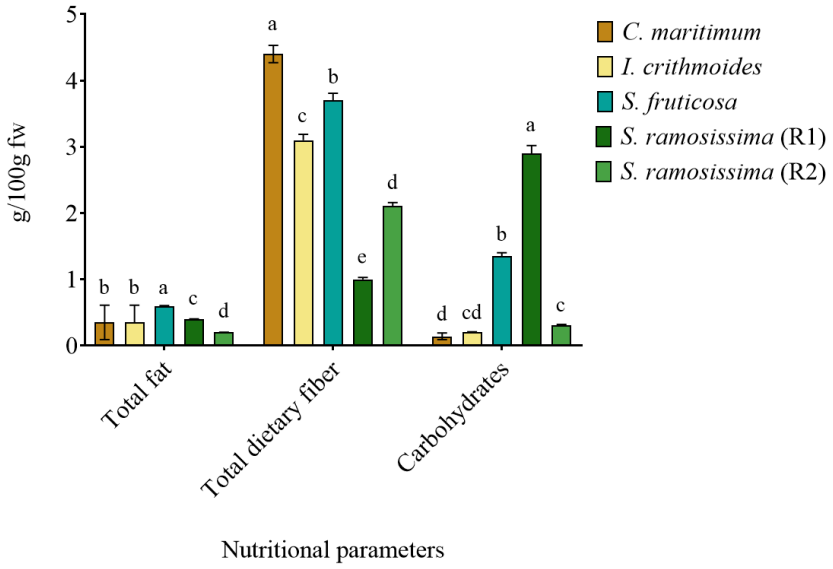

Figure 3.1 – Results summary of the nutritional parameters' total fat, total dietary fat, and carbohydrates, of halophyte plants. The letters (a-e) correspond to the statistical analysis performed to

calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

Considering the European Regulation (EC) Nº 1924/2006 [88], all the studied halophyte plants can be considered "rich in proteins", due to the fact that they show an energy value provided by proteins greater than 20% of the energy value of the plant. These plants can also be considered as having "low energy value", because they all show a lower energy value than 40 kcal/100g. Allegations such as "low fat content" and "no fat" are also possible in these plants, as they show values below 3 g/100g and 0.5 g/100g, respectively, of fat content. In addition, *C. maritimum*, *I. crithmoides* and *S. fruticosa* can be considered as "sources of fiber", because they show higher fiber values than 3 g/100g.

One of the main characteristics of these plants is the fact that they contain salt (NaCl) in their constitution and have a great tolerance to high concentrations of salt in the soil [3]. *S. ramosissima* (R1) showed the highest salt content value among the studied halophyte plants (5.62 g/100g fw). Considering that this halophyte plant is the only one that grew in the wild, and that the rest of the plants studied, including one of the same species, grow in a hydroponic environment, these differences may be justified based on the growth differences and type of soil.

The fatty acid profile of the halophyte plants was analyzed and is shown in **Table 3.1**. Halophyte plants present considerable levels of polyunsaturated fatty acids and the obtained results are in accordance with the literature [10]. All plants had a total value of polyunsaturated fatty acids higher than the total value of saturated fatty acids, *I. crithmoides* showing the highest PUFA/SFA ratio (2.97). The PUFA/SFA ratios observed in the plants of this work are in agreement with some ratios previously observed in the literature (ranging between 1.1 and 7.9) for other halophyte plants [84]. One of the known strategies for the prevention of cardiovascular diseases is to reduce the consumption of saturated fatty acids and focus on greater consumption of polyunsaturated fatty acids [89]. The main fatty acids present in the halophyte plants are palmitic, linoleic, and linolenic acids. Linoleic and linolenic acids are omega-6 and omega-3 fatty acids, respectively [84], and these fatty acids present several physiological functions, such as intervention in blood coagulation, and in inflammatory and immunological responses [90]. *S. ramosissima* (R2) showed the highest and *C. maritimum* the lowest content of omega-3 (46.5 and 23.5%, respectively), and the opposite for omega-6 (15.4 and 28.2%, respectively). Therefore, halophyte plants demonstrate interesting values that may allow a higher consumption of

polyunsaturated fatty acids, specially omega-3, and a reduced consumption in omega-6 and other saturated fatty acids in the diet, which has shown to be effective in decreasing the risk of cardiovascular diseases and cancers [89].

In addition to the nutritional parameters evaluated, the mineral composition of the halophyte plants was also analyzed. The results are shown in **Table 3.2** and expressed in mg/100g of fresh weight (fw).

Table 3.2 – Mineral composition of the halophyte plants and heavy metals quantification.

	C. maritimum	***I. crithmoides***	***S. fruticosa***	***S. ramosissima***	
Location	Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
Mineral composition (mg/100g fw)					
Sodium (Na)	570 ± 74^{c}	540 ± 70^{c}	1300 ± 169^{b}	2250 ± 293^{a}	600 ± 78^{c}
Calcium (Ca)	30.7 ± 3.7^{c}	82.0 ± 9.8^{a}	50.0 ± 6.0^{b}	31.8 ± 3.9^{c}	9.2 ± 1.1^{d}
Potassium (K)	159 ± 34^{c}	300 ± 63^{ab}	350 ± 74^{a}	105 ± 22^{c}	186 ± 39^{bc}
Phosphorus (P)	70.0 ± 6.3^{a}	60.0 ± 5.4^{a}	60.0 ± 5.4^{a}	$0.01 \pm 9x10^{-4b}$	0.027 ± 0.015^{b}
Iron (Fe)	0.7 ± 0.1^{c}	1.9 ± 0.3^{b}	3.5 ± 0.5^{a}	3.7 ± 0.05^{a}	0.1 ± 0.1^{c}
Magnesium (Mg)	13.30 ± 1.86^{c}	76.0 ± 10.6^{b}	52.0 ± 7.3^{b}	109.0 ± 15.3^{a}	66.0 ± 9.3^{b}
Manganese (Mn)	0.16 ± 0.02^{bc}	0.060 ± 0.008^{c}	0.47 ± 0.07^{a}	0.230 ± 0.032^{b}	0.57 ± 0.08^{a}
Zinc (Zn)	0.14 ± 0.02^{c}	0.42 ± 0.06^{b}	0.39 ± 0.05^{b}	0.62 ± 0.09^{a}	0.36 ± 0.05^{b}
Copper (Cu)	$0.007 \pm 6x10^{-4d}$	0.21 ± 0.02^{b}	0.38 ± 0.03^{a}	0.12 ± 0.01^{c}	0.12 ± 0.01^{c}
Heavy metals (mg/kg fw)					
Arsenic (As)	0.010 ± 0.002	0.004 ± 0.001	0.040 ± 0.001	0.040 ± 0.001	0.010 ± 0.001
Cadmium (Cd)	nd*	nd*	0.16 ± 0.03	nd*	nd*
Mercury (Hg)	nd**	nd**	nd**	nd**	nd**
Lead (Pb)	nd***	0.080 ± 0.004	0.10 ± 0.02	0.21 ± 0.02	0.040 ± 0.003

nd – not detected (limit of detection = 0.10*, 0.03**, 0.03*** mg/kg). Results are presented as mean ± SD. In each row, the letters (a-e) mean statistically significant differences ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

As previously described, *S. ramosissima* (R1) is the halophyte plant with the highest NaCl content and, consequently, it is the plant that has the significantly highest sodium content (2250 mg/100g fw). All plants from Faro show an identical composition in terms of sodium content (570, 540 and 600 mg/100g fw), with the exception of *S. fruticosa*, which shows a value of sodium content equal to 1300 mg/100g fw. In relation to other quantified minerals, it is also worth mentioning potassium, calcium and magnesium, since they have been studied as a viable alternative to rich in sodium salts [54,60]. *I. crithmoides*, *S. fruticosa* and *S. ramosissima* (R1) show the highest content in calcium (82.0 mg/100g fw), potassium (350 mg/100g fw), and magnesium (109.0 mg/100g fw), respectively. Despite the fact that sodium has the best correlation of saltier flavour with its increasing content, several other cations like K^+, Mg^{2+}, and Ca^{2+} can infer salty taste [91,92]. However, the taste of these ions can also be associated with bitterness, sourness, or astringency, especially potassium, which has been described as having a characteristic bitter taste [60,91,93].

Values previously reported [10] for *S. ramosissima*, also from Portugal, refer 8990, 892, 486, and 943 mg/100g of dried weight for sodium, potassium, calcium, and magnesium, respectively. Potassium and magnesium values are identical to the values reported in this work for *S. ramosissima* (R1), when converting the values, based on moisture, from fresh to dried weight (890 and 924 mg/100g, respectively). The differences in sodium and calcium (19068 and 269 mg/100g, respectively) may be related to the distinct location of the plant's growth zone, where, in the study cited, it was collected in the south of the country, while the one present in this work was collected in the north. Another review, from Italy, focusing on wild *C. maritimum*, showed a lower value of sodium content (291 mg/100g fw) and higher levels of potassium (335 mg/100g fw), magnesium (28 mg/100g fw), and calcium (310 mg/100g fw) [85], when compared to the values reported in **Table 3.2** for *C. maritimum*.

The quantification of heavy metals was important in this work. Salt marshes are natural deposits of heavy metals, which can accumulate in plants and animals, and lead to potential impacts in human health and safety [74]. The metals analyzed were arsenic, cadmium, mercury, and lead, and the results, expressed in mg/kg fw, are shown in **Table 3.2**. According to the Recommendation (EU) 2018/464, there is a need to establish maximum limit levels for these substances in seaweed and halophyte plants [94], but it was not yet possible to find a recent update to the publication. There is a mention of a

single limit value for mercury in seaweed and prokaryotic organisms equal to 0.01 mg/kg, however mercury was not detected in the studied halophyte plants, considering the limit of detection. Considering the lack of a new update, the regulation mentioned in the most recent one was followed, the European Regulation (EC) Nº 1881/2006. The maximum limits considered were between those established for fish and for leafy and stem vegetables, and aromatic herbs [95]. The cadmium and lead limits, within the mentioned categories, range between 0.05-0.20 and 0.10-0.30 mg/kg, respectively. No value for arsenic was found in the consulted regulation, however a scientific opinion on arsenic in food published by EFSA in 2010 mentions a value of 0.1 mg/kg for seafood [96]. The values obtained for the halophyte plants present in this work, therefore, comply with the consulted regulations.

3.2) Phytochemical composition

3.2.1) Total phenolic content

The results obtained for the total phenolic content (TPC) of the ethanolic halophyte extracts, using the Folin-Ciocalteu's assay, are presented in **Figure 3.2**. The results are expressed in fresh plant material (mg GAE/g fw).

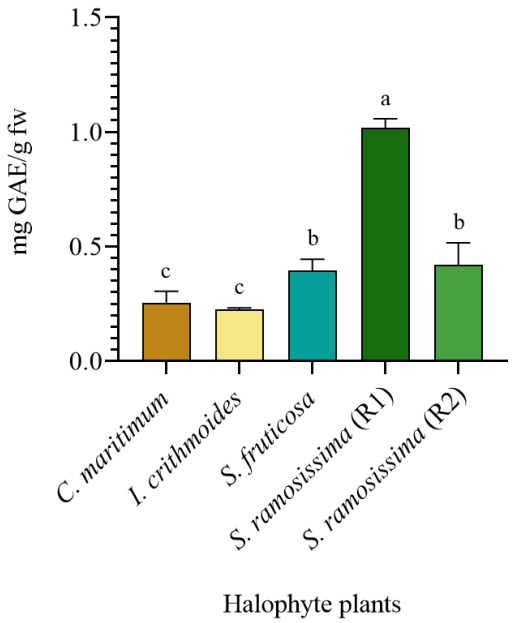

Figure 3.2 – Total phenolic content of selected halophyte plants. Each value represents mean ± standard deviation of triplicate extractions. The letters (a-c) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

The total phenolic content is primarily used to evaluate the antioxidant capacity from fruits, vegetables, and grains [78], because of the direct relationship between the known scavenging activity of phenolic compounds and the consequent minimization of the negative effects of oxidative stress [10]. Three groups of plants can be considered: *S. ramosissima* (R1) has the highest TPC value (1.02 mg GAE/g fw), followed by *S. fruticosa* and *S. ramosissima* (R2) (0.395 and 0.419 mg GAE/g fw, respectively), while *C. maritimum* and *I. crithmoides* have the lowest TPC values (0.255 and 0.224 mg GAE/g fw, respectively).

When comparing *S. ramosissima* from different growth environments, it was clear that the total phenolic content was different. *S. ramosissima* (R2) grew in a hydroponic system that provides a more controlled environment, while the *S. ramosissima* (R1) grew in a wild and, consequently, more hostile environment. It is known that this growth in a more stressful environment, subject to extreme conditions, allows plants to develop a more effective and powerful antioxidant system [97]. These different conditions experienced during growth can be the main cause for this difference between the TPC values obtained for the same halophyte plant species. However, in order to evaluate the importance of these results for bioactivity of these plants, results will be discussed in terms of the correlation between the TPC values and the results of the bioactivity tests performed later in this work.

A study from Tunisia with *C. maritimum* and *I. crithmoides* showed TPC values of 4.1-7.9 and 6.7-14.1 mg GAE/g of dried weight, respectively [12], while *C. maritimum* and *I. crithmoides* from this work displayed values of 2.28 and 2.29 mg GAE/g dw, respectively, when using the moisture value for conversion of fresh to dried weight. These differences can be justified not only due to the distinct plant growth location, but also with the different extraction solvent used, in which instead of using ethanol:water (80:20, v/v), acetone:water (80:20, v/v) was used. Studies have shown that better extraction yields and phenolic content are obtained with aqueous organic solvents, such as methanol, ethanol, and acetone [98–100]. However, higher antioxidant values are observed for aqueous ethanol extractions, when compared to methanol and acetone [100]. The use of these three organic solvents is possible and effective in the extraction of phenolic compounds and respective analyzes, however, ethanol seems to be the best option given the disadvantages of methanol use, such as inhalation and cutaneous toxicity [101,102]. *S. ramosissima* from France and other from Portugal showed values of TPC equal to 27.44

and 33.0 mg GAE/g dw, respectively [10,44], while *S. ramosissima* (R1) and (R2), in this study, demonstrated values of 8.62 and 4.82 mg GAE/g dw, respectively, after conversion. The use of different solvents and extraction methods and the different location of plant growth may be one of the factors that contribute to these different TPC values. In addition, changes in the environment and climate may interfere in the chemical composition of the plants, due to the known relationship between the production of phenolic compounds and the stress experienced during the plant's growth [103].

3.2.2) Phenolic compounds identification by LC-DAD-ESI-MS/MS

The phenolic compounds of the halophyte plants were identified comparing their fragmentation pattern using negative ionization mode with the data found in the literature. Considering that, at different wavelengths, there were no new verified peaks, the chromatogram at 280 nm was chosen, since it is the most general detection wavelength used for the simultaneous determination of different phenolic compounds [104]. The obtained chromatograms on the diode array of the LC-DAD-ESI-MS/MS at 280 nm for each plant are shown in the **Figure 3.3**. Sixty-two compounds were putatively identified in the chromatograms from the four different species (**Appendix B**), in which six are organic acids, thirty-two are phenolic acids (including hydroxybenzoic and hydroxycinnamic acids), and twenty-one are flavonoid compounds.

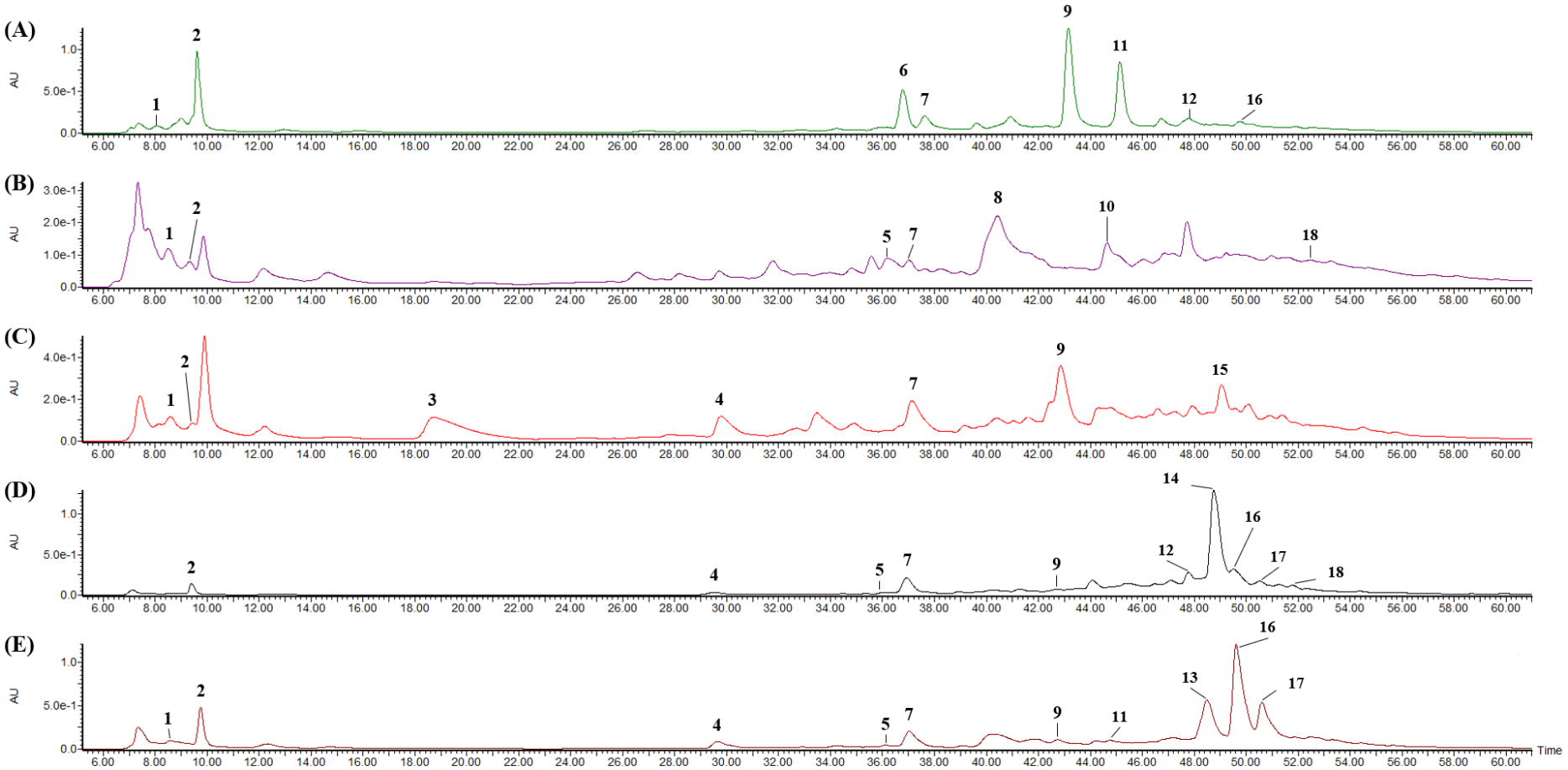

Figure 3.3 – LC-DAD-ESI-MS/MS chromatograms at 280 nm for extracts of *C. maritimum* (A), *I. crithmoides* (B), *S. fruticosa* (C), and *S. ramosissima* (R1) (D) and (R2) (E). The numbers represent the compounds in common or those with the most intense peaks: 1- malic acid, 2- quinic acid, 3- *p*-coumaric acid derivative, 4- 3-caffeoylquinic acid, 5- *p*-coumaric acid, 6- *p*-coumaric acid-glucoside, 7- 5-caffeoylquinic acid, 8- pinobanksin-5-methyl ether-3-acetate, 9- *p*-coumaroylquinic acid (isomer 1), 10- feruloylquinic acid, 11- *p*-coumaroylquinic acid (isomer 2), 12- quercetin-3-hexoside, 13- 3,4-dicaffeoylquinic acid, 14- quercetin-malonyhexoside, 15- isorhamnetin-3-rabinobioside, 16- 3,5-dicaffeoylquinic acid, 17- 4,5-dicaffeoylquinic acid, 18- caffeic acid-glucuronide-glucoside.

Some organic acids were identified in all halophyte plants, such as malic (compound **1**) and quinic (compound **2**) acids, with $[M-H]^-$ ions at *m/z* 133 and 191, respectively, according to their fragmentation products [103,105]. In addition, citric acid was also identified in *C. maritimum,* with a $[M-H]^-$ ion at *m/z* 191 and fragment ions at *m/z* 111, 87 and 85, which is an identical fragmentation pattern to quinic acid [103]. The distinction between the two compounds was possible due to the retention times, because citric acid has a lower retention time than quinic acid [106].

Hydroxycinnamic acids were identified in the halophyte plant extracts, such as *p*-coumaric acid (compound **5**), which was present in *I. crithmoides* and both *S. ramosissima*, with an $[M-H]^-$ ion at 163 and characterized by the presence of product ion 119 [35,107].

The presence of *p*-coumaric acid derivatives was detected in the halophyte plant extracts, such as *p*-coumaric acid-glucoside in *C. maritimum* (compound **6**) [35,107,108], with a $[M-H]^-$ ion at *m/z* 325, and a not identified *p*-coumaric derivative in *S. fruticosa* (compound **3**), with a product ion at *m/z* 261. In addition, in the extract of *S. fruticosa*, other derivatives of *p*-coumaric acids were detected at *m/z* 163, but were not identified [35,107]. Compounds **9** and **11**, showing a $[M-H]^-$ ion at *m/z* 337, were identified as *p*-coumaroylquinic acids, due to the characteristic fragments at *m/z* 191 and 163 [109]. In several studies [110–112], *p*-coumaric acid has shown antioxidant properties.

Compounds **4** and **7** were identified as 3-caffeoylquinic and 5-caffeoylquinic acids, respectively. These phenolic acids, characterized by the negative ion at *m/z* 353, were both identified in *S. fruticosa* and both *S. ramosissima*, but only 5-caffeoylquinic acid was identified in all of the studied plants. These compounds, when both present, are possible to distinguish due to their different retention times and the difference in fragments relative intensities, such as fragment at *m/z* 179 which is generally more intense in 3-caffeoylquinic acid [113,114]. In addition, a 3-caffeoylquinic acid standard was later used and this identification was confirmed. 3-, 4-, and 5-caffeoylquinic acid (also known as chlorogenic acid) isomers and their derivatives not only have a well-known antioxidant activity [115,116], but also antimicrobial, hepatoprotective, anti-inflammatory, antipyretic, antiviral, anti-obesity, and antihypertensive [117,118]. In addition, caffeoylquinic dimers were also identified, such as 3,4-dicaffeoylquinic (compound **13**), 3,5-dicaffeoylquinic (compound **16**), and 4,5-dicaffeoylquinic acids (compound **17**), with

a $[M-H]^-$ ion at *m/z* 515 and fragments characteristics of caffeoylquinic, quinic, and caffeic acids (*m/z* 353, 191, and 179, respectively) [35,105].

Other high intensity peaks were observed in some halophyte plants, in particular, quercetin derivatives, such as quercetin-3-hexoside (compound **12**) and quercetin-malonyhexoside (compound **14**) (negative ions at *m/z* 463 and 549, respectively), identified in *C. maritimum* and *S. ramosissima* (R1). Both flavonol derivatives show a product ion at *m/z* 301, referring to quercetin [35,113]. Quercetin has shown antioxidant, anti-carcinogenic, antiviral, antibacterial, and anti-inflammatory activities [119,120].

I. crithmoides presents, in the chromatogram, high peaks that were identified as pinobanksin-5-methyl ether-acetate (compound **8**) and feruloylquinic acid (compound **10**), with $[M-H]^-$ ions at *m/z* 327 [121] and 367 [122], respectively. Another flavonoid glycoside identified was isorhamnetin-3-robinobioside (compound **15**) [123], detected in a higher concentration in the *S. fruticosa* extract. In addition, a caffeic acid derivative identified as caffeic acid-glucuronide-glucoside (compound **18**) [124] is present in both *I. crithmoides* and *S. ramosissima* (R1).

The relative areas (in percentage) of the chromatograms' peaks of the compounds shown in **Figure 3.3** were calculated and are shown in **Table 3.3**.

Table 3.3 – Areas (%) observed for main peaks of the halophyte plant chromatograms by LC-DAD-ESI-MS/MS at 280 nm.

			C. maritimum	***I. crithmoides***	***S. fruticosa***	***S. ramosissima***	
Location			Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
Peak (Fig. 7)	**Compound**	**Class**			**Area %**		
1	Malic acid	Organic acid	2.04	2.85	2.17	1.03	2.54
2	Quinic acid	Organic acid	15.43	1.42	0.83	1.63	4.50
3	*p*-coumaric acid derivative	Phenolic acid	-	-	6.40	-	-

			C. maritimum	*I. crithmoides*	*S. fruticosa*	*S. ramosissima*	
Location			Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
4	3-caffeoylquinic acid	Phenolic acid	-	-	3.42	0.95	1.83
5	*p*-coumaric acid	Phenolic acid	-	2.39	-	0.10	0.42
6	*p*-coumaric acid-glucoside	Phenolic acid	8.59	-	-	-	-
7	5-caffeoylquinic acid	Phenolic acid	4.16	1.62	5.45	5.69	3.85
8	pinobanksin-5-methyl ether-3-acetate	Flavo-noid	-	14.29	-	-	-
9	*p*-coumaroyl-quinic acid (isomer 1)	Phenolic acid	21.59	-	9.11	0.07	2.20
10	feruloylquinic acid	Phenolic acid	-	4.93	-	-	-
11	*p*-coumaroyl-quinic acid (isomer 2)	Phenolic acid	12.80	-	-	-	2.46
12	quercetin-3-hexoside	Flavo-noid	2.95	-	-	8.58	-
13	3,4-dicaffeoylqui-nic acid	Phenolic acid	-	-	-	-	13.64
14	quercetin-malonyhexosi-de	Flavo-noid	-	-	-	56.88	-
15	isorhamnetin-3-rabinobioside	Flavo-noid	-	-	4.19	-	-

			C. maritimum	*I. crithmoides*	*S. fruticosa*	*S. ramosissima*	
Location			Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
16	3,5-dicaffeoylquinic acid	Phenolic acid	1.53	-	-	6.50	20.89
17	4,5-dicaffeoylquinic acid	Phenolic acid	-	-	-	4.37	12.28
18	caffeic acid-glucuronide-glucoside	Phenolic acid	-	2.45	-	2.19	-
Σ (caffeic acids and derivatives) [a,b]			-	6.76	3.39	2.19	-
Σ (ferulic acids and derivatives) [a]			-	4.93	-	0.45	6.32
Σ (*p*-coumaric acids and derivatives) [a]			43.81	3.90	26.79	0.17	5.08
Σ (caffeoylquinic acids and derivatives) [a]			5.69	1.62	11.16	17.51	52.49
Σ (quercetin and derivatives) [a]			4.59	-	2.16	73.33	-

[a] The sum of the areas may involve values that are not shown in this table of main compounds, but that were still calculated for compounds detected and identified in **Appendix B**.
[b] Except caffeoylquinic acids.

A high total area of compounds derived from *p*-coumaric is demonstrated in *C. maritimum* (43.81%) and *S. fruticosa* (26.79%). However, *S. ramosissima* (R1) shows a much higher total relative area for quercetin and its derivatives (73.33%) and *S. ramosissima* (R2) for caffeoylquinic acids and their derivatives (52.49%). It is noted that the halophyte plants studied have different profiles and, therefore, different results are expected in relation to the bioactivity assays to be carried out later in this work.

3.3) Bioactivity

3.3.1) Antioxidant activity

The antioxidant activity of the halophyte plant extracts was measured by oxygen radical absorbance capacity (ORAC) and hydroxyl radical scavenging capacity (HOSC) assays and the results are shown in **Figure 3.4**. The results are expressed as fresh plant material (μmol TEAC/g fw).

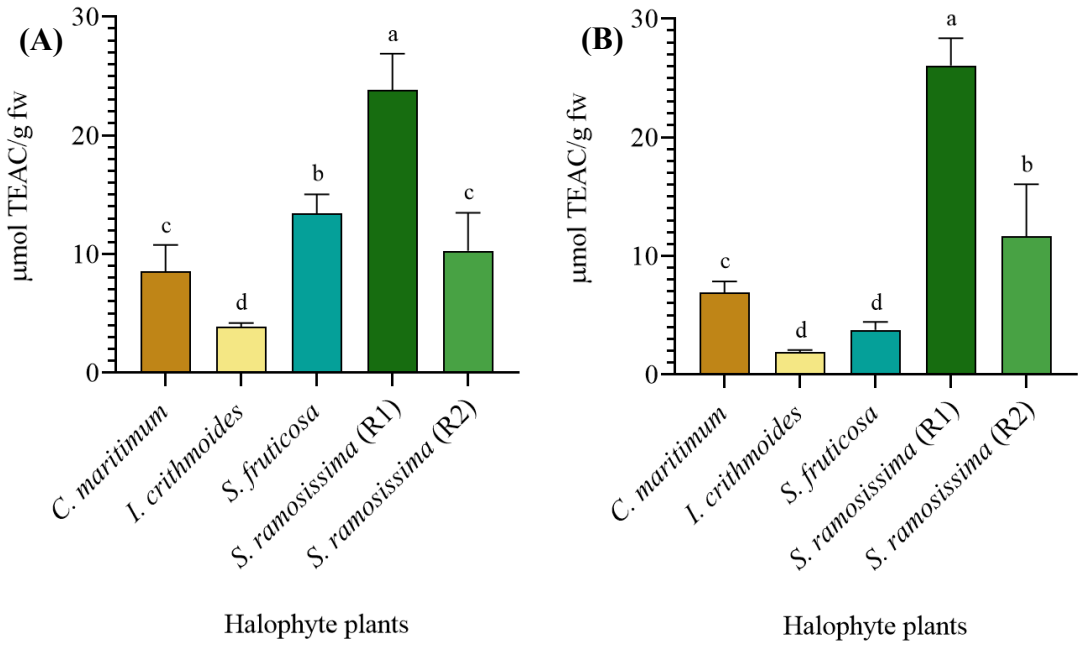

Figure 3.4 – Antioxidant activity assays ORAC (A) and HOSC (B) for the halophyte plants. Each column represents mean ± standard deviation of triplicate extractions. The letters (a-d) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

Both methods were used to assess antioxidant activity due to their fundamental difference: ORAC evaluates the antioxidant capacity towards peroxyl radicals, which are the most prevalent free radical in human biology, and HOSC evaluates the scavenging capacity for hydroxyl radical, which is the most reactive species generated in biological systems [82,103]. This allows the extracts to be evaluated for their antioxidant capacity for different radical oxygen species.

The halophyte plant *S. ramosissima* (R1) demonstrated a high and similar value of antioxidant activity for both ORAC and HOSC methods (23.8 and 26.1 µmol TEAC/g fw, respectively), as well as *C. maritimum*, *I. crithmoides* and *S. ramosissima* (R2) (8.51 and 6.89, 3.88 and 1.89, and 10.3 and 11.6 µmol TEAC/g fw, respectively). However, a rather different value of antioxidant capacity between ORAC and HOSC is observed for *S. fruticosa* (13.4 and 3.77 µmol TEAC/g fw, respectively). This means that *S. fruticosa* is more efficient to scavenge peroxyl radicals than to scavenge hydroxyl radicals [82,125]. The correlation between both methods and the TPC values is shown later in this work.

Phenolic acids present in these plants, such as *p*-coumaric acids and its derivatives, and caffeic acids and its derivatives, have shown a high capacity for scavenging free radicals

[112,116]. Chlorogenic acid and its derivatives, and flavonoids such as quercetin and its derivatives, were also identified in the halophyte plants, and their antioxidant activity is well-known in the literature [115,116,119]. *S. ramosissima* (R1) shows a profile of phenolic compounds predominant in derivatives of quercetin (73.33%) and, consequently, the highest antioxidant activity among the halophyte plants studied. It can be concluded that it is due to the considerable presence of quercetin derivatives that this halophyte plant shows high antioxidant activity. The same can be observed in *S. ramosissima* (R2), which displays a high percentage of chlorogenic acid derivatives (52.49%). This halophyte plant showed an interesting antioxidant activity for both methods. These profiles confirm the values and, consequently, the effectiveness in the *in vitro* antioxidant assays in relation to the antioxidant capacity of these halophyte plants.

In literature, a concentrated extract of *S. ramosissima* from France showed an ORAC value of 9060 µmol TEAC/g dw [44], which, compared to the value of the plant studied in this work (201.7 µmol TEAC/g dw, after conversion based on the moisture value) is higher. However, this considerable difference can be explained with not only the distinct extraction methods, but also due to environmental growth differences that may affect the halophyte plant's phytochemical profile [103], as mentioned earlier in the TPC values' discussion.

3.3.2) Antihypertensive activity

The ACE inhibition fluorometric assay was performed to assess the antihypertensive activity of halophyte plants. ACE inhibition is directly related to the decrease in blood pressure, and therefore assays to test ACE inhibitors serve to assess the antihypertensive activity that certain compounds or samples may have [126]. Different concentrations (0-500 mg/ml) were tested to be able to form an inhibition curve in relation to the concentrations and calculate the half maximal inhibitory concentration (IC_{50}). The calculated IC_{50} values for each halophyte plant, expressed in mg/ml, are shown in **Figure 3.5**. In addition, a known angiotensin-converting enzyme inhibitor drug, lisinopril, was used as a positive control and to evaluate the effectiveness of the method. The curve with the different concentrations of lisinopril tested to calculate its IC_{50} is shown in **Figure 3.6**.

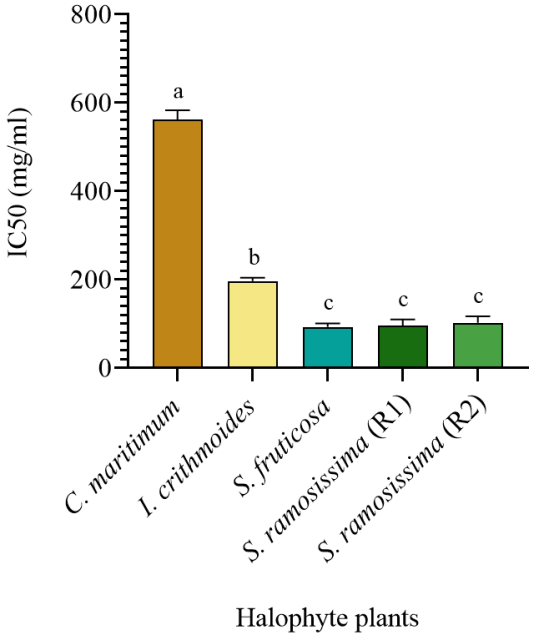

Figure 3.5 – ACE inhibitory assay IC_{50} results of tested halophyte plants. Each value represents mean ± standard deviation of duplicates. The letters (a-c) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

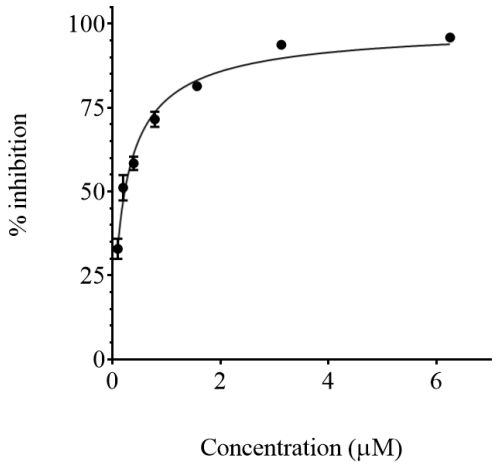

Figure 3.6 – Lisinopril curve with the percentage of ACE inhibition as a function of the concentration in µM.

A higher calculated IC_{50} value for the inhibitory activity towards ACE means a worse antihypertensive activity. *S. fruticosa* and *S. ramosissima* (R1) and (R2) show significantly similar and low IC_{50} values (93.0, 95.6 and 102.3 mg/ml, respectively). *I. crithmoides* displays a worse antihypertensive activity than the previous three mentioned halophyte plants due to its higher IC_{50} value (197 mg/ml), while *C. maritimum* demonstrates the significantly highest IC_{50} value (562 mg/ml) and, consequently, the

worst antihypertensive activity among the studied halophyte plants. The IC_{50} for lisinopril is 0.224 μM, which is roughly $9.08x10^{-5}$ mg/ml. Given the fact that the antihypertensive efficacy of lisinopril is known and is one of the most effective when tested and compared to other antihypertensive drugs like captopril, zofenopril, enalapril, ramipril, and fosinopril [127], and that an IC_{50} value of $4.72x10^{-5}$ mg/ml was previously reported [128], we can conclude that the method worked correctly and that the results are reliable.

The tested halophyte plants are rich in phenolic compounds, in particular, flavonoids and phenolic acids, that have been described as good ACE inhibitors and, consequently, showing antihypertensive activity, such as quercetin derivatives [65,66], caffeoylquinic acid derivatives [129], and ferulic acid derivatives [129,130]. *C. maritimum* showed the significantly lowest antihypertensive activity despite its high percentage of *p*-coumaric acid derivatives (43.81%). On the other hand, *S. fruticosa* and *S. ramosissima* (R1) and (R2) showed low and significantly identical IC_{50} values. A percentage of 73.33% relative area for quercetin derivatives in *S. ramosissima* (R1) and 52.49% for caffeoylquinic acids and their derivatives in *S. ramosissima* (R2) was verified. As previously stated, these compounds have antihypertensive activity reported in the literature, which justifies the IC_{50} values obtained in the ACE inhibitory assay.

Some medicinal plants (*Phalleria macrocarpa*, *Gynura procumbens*, *Melia azedarach*, *Hibiscus rosasinensis*, and others), in Indonesia, showed IC_{50} values for methanolic extracts between 0.102-0.483 mg/ml [131]. These plants were characterized by the presence of compounds that have reported ACE inhibitory activity, such as tannins, proanthocyanidins, flavonoids, fatty acids, terpenoids, alkaloids, oligosaccharides, and peptide amino acids. Although the studied halophyte plants have higher values than this range, *S. fruticosa* and *S. ramosissima* (R1) and (R2) still present an interesting antihypertensive activity to be explored.

The Pearson correlations between the total phenolic content and the methods used to assess the antioxidant activity, ORAC and HOSC, and antihypertensive activity, ACE inhibitory assay, of the halophyte plants are shown in **Table 3.4**. For ORAC and HOSC methods, a very high positive Pearson correlation ($r > 0.90$) [132] with the TPC values was obtained ($r = 0.9575$ and $r = 0.9503$, respectively). In addition, the correlation between ORAC and HOSC was made, in which a high positive Pearson correlation was obtained ($r = 0.8831$), which means that the studied halophyte plants have a good ratio of compounds capable of scavenging both peroxyl and hydroxyl radicals [82,103].

However, a low negative Pearson correlation is obtained between the ACE inhibitory assay and the TPC values (r = -0.4652), which also implied a low correlation with ORAC (r = -0.4083) and HOSC (r = -0.2909). This lower correlation is justified by the fact that maybe only some of the phenolics quantified in the TPC value demonstrate antihypertensive activity. A study of the synergistic effect between the identified compounds isolated would be a good strategy to understand the main phenolic compounds responsible for the antihypertensive activities demonstrated by the halophyte plants studied and the correlations obtained in **Table 3.4**. In addition, other compounds not focused in this work, such as peptide and/or amino acids, may be present in the evaluated extracts and contribute to antihypertensive activity, as demonstrated in the literature [131,133].

Table 3.4 – Pearson (r) correlations calculated for the total phenolic content values with the biological activity values observed in the halophyte plants extracts.

	C. maritimum	***I. crithmoides***	***S. fruticosa***	***S. ramosissima***	
Location	Faro	Faro	Faro	Aveiro (R1)	Faro (R2)
Results summary					
TPC (mg GAE/g fw)	0.255	0.224	0.395	1.02	0.419
ORAC (µmol TEAC/g fw)	8.51	3.88	13.4	23.8	10.3
HOSC (µmol TEAC/g fw)	6.89	1.89	3.77	26.1	11.6
ACE inhibitory assay (mg/ml)	561.5	196.8	93	95.6	102.3
Pearson correlations (r)*					
TPC vs. ORAC	0.9575 (significant)				
TPC vs. HOSC	0.9506 (significant)				
ORAC vs. HOSC	0.8831 (significant)				
TPC vs. ACE inhibitory assay	-0.4652 (not significant)				
ACE inhibitory assay vs. ORAC	-0.4083 (not significant)				
ACE inhibitory assay vs. HOSC	-0.2909 (not significant)				

* Correlation is significant at the 0.05 level (2-tailed).

Part 2: Dried *S. ramosissima* as a natural ingredient - Impact of drying process on the nutritional value, phytochemical profile, and bioactivity of the plant

S. ramosissima from a natural environment (R1) showed antihypertensive activity and the highest antioxidant activity compared to the other halophyte plants. In addition, the phenolic profile of this halophyte plant was mostly based on quercetin derivatives, which is a flavonoid that has shown several interesting biological activities in literature, beyond the ones studied in this work, such as anti-obesity, anti-carcinogenic, antiviral, antibacterial, and anti-inflammatory [120]. Considering these aspects, this halophyte plant was selected to apply as an ingredient in food, but first to proceed with the two drying processes (drying at 70 ºC and lyophilization) and evaluate their impact on the nutritional parameters and phytochemical content.

3.4) Nutritional profile and mineral composition

The nutritional parameters between the dried at 70 ºC and lyophilized *S. ramosissima* (R1) were compared and are shown in **Table 3.5**.

Table 3.5 – Nutritional and fatty acids profile of the fresh, dried at 70 ºC and lyophilized *S. ramosissima* (R1).

	S. ramosissima		
Location	Aveiro (R1)		
Plant material processing	Fresh	Dried	Lyophilized
Nutritional composition (g/100g dw)			
Moisture	88.20 ± 0.88^{a}	7.66 ± 0.08^{b}	3.20 ± 0.03^{c}
Proteins	13.50 ± 0.51*^{a}	8.48 ± 0.34^{c}	11.00 ± 0.44^{b}
Total fat	3.39 ± 0.03*^{a}	1.20 ± 0.01^{c}	1.80 ± 0.02^{b}
Ashes	50.10 ± 2.04*^{a}	41.00 ± 1.64^{b}	44.20 ± 1.77^{b}
Total dietary fiber	8.47 ± 0.26*^{c}	29.00 ± 0.87^{a}	26.90 ± 0.81^{b}
Carbohydrates	24.60 ± 1.02*^{a}	12.70 ± 0.51^{b}	12.90 ± 0.52^{b}
Energy (kcal/100g dw)	200.0 ± 8.0*^{a}	154.0 ± 6.1^{c}	166.0 ± 6.6^{b}
Salt	47.6 ± 6.2*^{a}	36.8 ± 4.8^{ab}	28.5 ± 3.7^{b}
Fatty acids profile (%)			
Palmitic acid (C16:0)	24.00 ± 0.01^{b}	26.80 ± 0.01^{a}	19.80 ± 0.01^{c}
Stearic acid (C18:0)	2.00 ± 0.01^{a}	1.90 ± 0.01^{b}	1.30 ± 0.01^{c}
Oleic acid (C18:1)	4.20 ± 0.01^{a}	3.50 ± 0.01^{b}	1.60 ± 0.01^{c}
Linoleic acid (C18:2)	24.00 ± 0.01^{b}	23.70 ± 0.01^{c}	28.00 ± 0.01^{a}

	S. ramosissima		
Location	Aveiro (R1)		
Plant material processing	Fresh	Dried	Lyophilized
Linolenic acid (C18:3)	33.30 ± 0.01^{b}	27.70 ± 0.01^{c}	40.20 ± 0.01^{a}
Arachidic acid (C20:0)	0.90 ± 0.01^{b}	1.20 ± 0.01^{a}	0.80 ± 0.01^{c}
Eicosenoic acid (C20:1)	nd	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}
Behenic acid (C22:0)	1.40 ± 0.01^{c}	4.20 ± 0.01^{a}	2.10 ± 0.01^{b}
Lignoceric acid (C24:0)	1.70 ± 0.01^{c}	2.90 ± 0.01^{a}	2.50 ± 0.01^{b}
Σ SFA	34.30 ± 0.01^{b}	42.60 ± 0.01^{a}	28.10 ± 0.01^{c}
Σ MUFA	7.30 ± 0.01^{a}	4.80 ± 0.01^{b}	3.10 ± 0.01^{c}
Σ PUFA	58.40 ± 0.01^{b}	52.70 ± 0.01^{c}	69.20 ± 0.01^{a}
PUFA/SFA	1.70 ± 0.01^{b}	1.24 ± 0.01^{c}	2.46 ± 0.01^{a}

* The values were converted from fresh weight to dried weight, according to the moisture value.
nd – not detected (limit of quantification = 0.05 g/100g), SFA – total saturated fatty acids, MUFA – total monounsaturated fatty acids, PUFA – total polyunsaturated fatty acids. Results are presented as mean ± SD. The letters (a-c) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

The lyophilization process was more effective in removing water from the plant matrix, observing a significantly lower moisture percentage when compared to the dried at 70 ºC value (3.20 and 7.66%, respectively). These differences in the amount of water present in the plant may affect the remaining nutritional parameters values. The contents of ashes, carbohydrates, and salt do not demonstrate a significant difference between the two drying methods. However, the situation is not the same when comparing the content of proteins and fat. Lyophilized *S. ramosissima* showed a higher protein and fat content (11.00 and 1.80 g/100g dw, respectively) than the dried plant (8.48 and 1.20 g/100g dw, respectively). Proteins and fat may suffer denaturation at temperatures above 40 ºC, and oxidation, respectively [134,135]. These changes in protein and fat content are visible in the results obtained, where the lyophilized plant, due to the drying process that involves low temperature and vacuum atmosphere, is able to prevent the degradation of these same macronutrients.

The values of the fresh plant converted into dried weight are also shown in the **Table 3.5**. It is observed that, despite the associated error in the conversion of the values, given the different fresh matrix when compared to the plants in a dried state, the content of ashes, carbohydrates, and salt is very different in the fresh plant when compared to the dried and lyophilized plant. Halophyte plants may accumulate soluble carbohydrates in response to salt stress [84]. Given this and taking into consideration these compounds solubility, it

can be justified the presence of a higher content of ashes, carbohydrates, and salt in the fresh plant (50.1, 24.6, and 47.6 g/100g dw, respectively) when compared to the dried and lyophilized plant (41.0, 12.7 and 36.8, and 44.2, 12.9 and 28.5 g/100g dw, respectively).

The profile of the main fatty acids was also compared between the two drying methods (**Table 3.5**). It is possible to observe that the lyophilized plant has the highest amount of PUFA (69.2%) and the lowest amount of SFA (28.1%), whereas the dried plant shows the lowest PUFA value (52.7%) and the highest SFA value (42.6%). Studies have demonstrated that temperature has effects on the fatty acid profile [136,137]. Linoleic and linolenic acids are strongly affected with an elevated temperature, with a reduction of their content. This reduction is related to the effect of the temperature on fatty acids desaturases, which prevents the desaturation of saturated fatty acids [137,138]. The same can be seen when comparing the significantly different linoleic and linolenic acid values of the dried plant (23.7 and 27.7%, respectively) with the lyophilized plant (28.0 and 40.2%, respectively). In addition, it is also known that the oleic acid content increases with increasing temperature [136], which is also possible to observe when comparing the dried and lyophilized content (3.5 and 1.6%, respectively). These results demonstrate that lyophilization is the best process for a more suitable for health fatty acid profile, due to its observed higher PUFA/SFA ratio (2.46).

3.5) Phytochemical composition

3.5.1) Total phenolic content

The values of total phenolic content (TPC) obtained in the extracts for fresh (converted in dried weight), dried at 70 ºC and, lyophilized *S. ramosissima* (R1) were compared (**Figure 3.7**). The results are expressed in dried plant material (mg GAE/g dw).

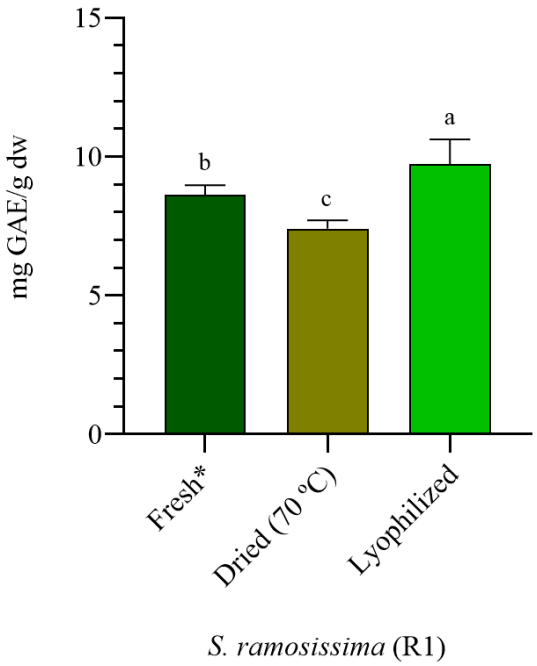

Figure 3.7 – Total phenolic content for fresh, dried at 70 ºC, and lyophilized *S. ramosissima* (R1). Each column represents mean ± standard deviation of triplicate extractions. The letters (a-c) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.
* The values were converted from fresh weight to dried weight, according to the moisture value.

From the results obtained, a higher TPC value was observed in the lyophilized *S. ramosissima* extract than in the dried plant extract (9.74 and 7.41 mg GAE/g dw, respectively). These values may be explained by the use of drying processes that involve high temperatures, such as 70 ºC, that can lead to a reduction in the total phenolic content due to oxidation and decomposition of these bioactive compounds [139]. For instance, quinic acid, which is an organic compound that is part of the structure of phenolic acids identified in *S. ramosissima* as caffeoylquinic acids and *p*-coumaroylquinic acids [105], may experience an increase in its concentration due to the breakdown of these same phenolic acids mentioned [140]. This same fact will be confirmed and studied later.

On the other hand, a drying process that involves low temperatures and vacuum, such as lyophilization, can prevent the decomposition of the bioactive compounds due to the existence of a limited atmosphere in oxygen and low temperature [141]. This can prevent the oxidation of phenolic compounds, which can justify the higher value of TPC in the lyophilized halophyte extract when compared to the dried at 70 ºC plant extract.

3.5.2) Phenolic profile comparison of the 3 different plant processing (fresh, dried, and lyophilized) by LC-DAD-ESI-MS/MS and main phenolic compounds quantification by LC-DAD

The extracts of fresh, dried, and lyophilized *S. ramosissima* were analyzed on LC-DAD-ESI-MS/MS and the profile of the obtained chromatograms was compared (**Figure 3.8**). In addition, the individual identification of each extract was also made (**Appendix C**), so that, if possible, due to the concentration of the compounds due to the drying process, identify new compounds that were not possible to identify in the previous extract of the fresh plant.

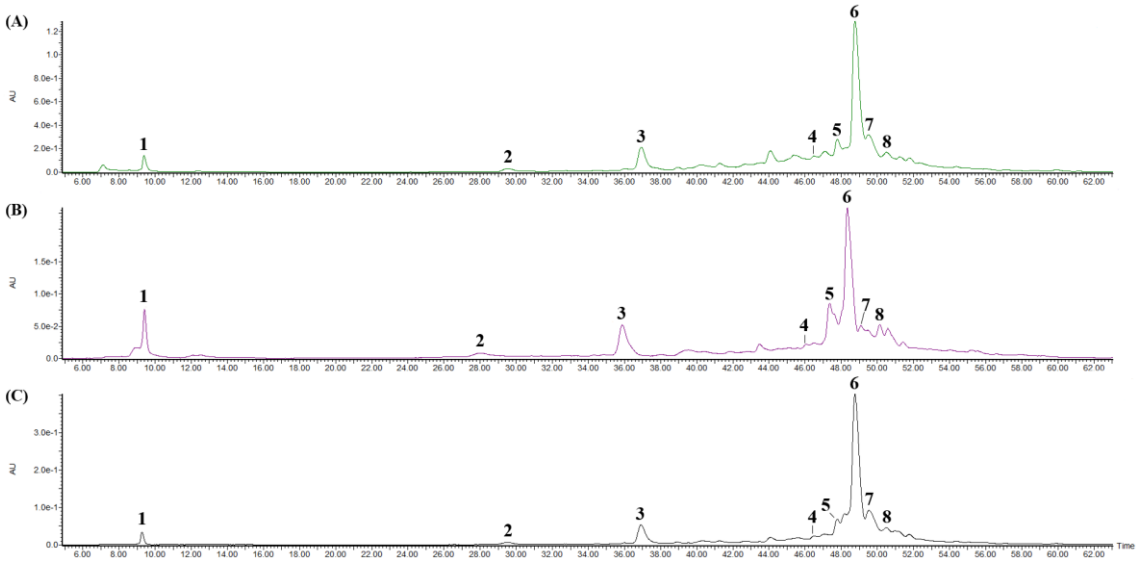

Figure 3.8 – LC-DAD-ESI-MS/MS chromatograms at 280 nm for fresh (A), dried at 70 ºC (B), and lyophilized (C) *S. ramosissima* (R1). The numbers represent the following compounds: 1- quinic acid, 2- 3-caffeoylquinic acid, 3- 5-caffeoylquinic acid, 4- quercetin-rhamnosyl-hexoside, 5- quercetin-3-hexoside, 6- quercetin-malonyhexoside, 7- 3,5-dicaffeoylquinic acid, and 8- 4,5-dicaffeoylquinic acid.

It is possible to notice, in the chromatograms presented in **Figure 3.8**, that the extract of the lyophilized plant extract is able to maintain a profile more identical to the fresh plant than the dried plant extract. However, in the dried plant extract, there are slight changes to the general profile, namely in the peak's intensity identified as quinic acid and 5-caffeoylquinic acid. In addition to the differences and profile changes, it was possible to identify some compounds that had not been identified in the fresh plant, such as a second isomer of *p*-coumaroylquinic acid and a *p*-coumaric acid benzyl ester derivative in the dried plant, and a hydrocaffeoylquinic acid and another isomer of caffeic acid-glucuronide-glucoside in the lyophilized plant (**Appendix C**).

To confirm these changes in the profile, some compounds were quantified, namely quinic acid, caffeoylquinic acids and their derivatives, and quercetin and its derivatives. The quantification was performed in LC-DAD and the results are shown in the **Table 3.6**. A mix with standards (0.78-100 ppm) was used for the quantification of the compounds which included: gallic acid, 3-caffeoylquinic acid, quercetin-3-hexoside, and quercetin-3-acetylhexoside (**Appendix D**).

Table 3.6 - Compounds quantified in the fresh, dried, and lyophilized *S. ramosissima* (R1).

			S. ramosissima
Location		Aveiro (R1)	
Plant material processing	Fresh*	Dried	Lyophilized
Quantified compounds (µg/g dw)			
Quinic acid	63.96 ± 14.80^{b}	117.40 ± 1.28^{a}	52.79 ± 1.48^{b}
3-caffeoylquinic acid	126.5 ± 32.1^{a}	136.10 ± 2.51^{a}	99.43 ± 3.13^{a}
5-caffeoylquinic acid	369.3 ± 88.9^{a}	318.7 ± 11.1^{a}	422.0 ± 13.4^{a}
Quercetin-rhamnosyl-hexoside	60.02 ± 13.6^{ab}	52.91 ± 1.34^{b}	88.04 ± 2.59^{a}
Quercetin-3-hexoside	405.4 ± 66.9^{b}	688.9 ± 16.5^{a}	617.4 ± 19.8^{a}
Quercetin-malonyhexoside	2458.0 ± 605.2^{b}	1578 ± 30^{b}	4281.0 ± 24.1^{a}
3,5-dicaffeoylquinic acid	219.2 ± 0.8^{b}	223.4 ± 9.3^{b}	480.6 ± 15.6^{a}
4,5-dicaffeoylquinic acid	96.41 ± 9.03^{b}	223.2 ± 9.7^{a}	252.40 ± 7.57^{a}

* The values were converted from fresh weight to dried weight, according to the moisture value.
Results are presented as mean ± SD. The letters (a and b) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

Phenolic compounds such as quercetin-rhamnosyl-hexoside, quercetin-malonyhexoside, and 3,5-dicaffeoylquinic acid show a higher and significantly different value in the lyophilized plant (88.04, 4281.0 and 480.6 µg/g dw, respectively) when compared to the fresh and dried plant (60.02, 2458.0 and 219.2 µg/g dw, and 52.91, 1578.0 and 223.4 µg/g dw, respectively). However, 3- and 5-caffeoylquinic acids do not show a significant value difference when comparing the three different halophyte plant material extracts.

The higher concentrations observed in the lyophilized extract may be mainly due to the known effect that temperature has on phenolic compounds, such as flavonoids and phenolic acids, and their consequent degradation [142]. Quinic acid was quantified for this reason. Quinic acid, along with caffeic acid, is part of both caffeoylquinic acids and their dimers (**Figure 3.9**) [105]. The quinic acid quantification had as a goal to allow to

see if the temperature of 70 ºC would influence the phenolic acids and its consequent breakage into smaller phenolic and organic acids, and it is noticed that almost twice the concentration of quinic acid in the fresh (63.96 µg/g dw) and lyophilized (52.79 µg/g dw) plant extract is observed in the dried plant (117.40 µg/g dw). In conclusion, an identical and more concentrated profile, when compared to the fresh halophyte plant, is observed in the lyophilized plant extract.

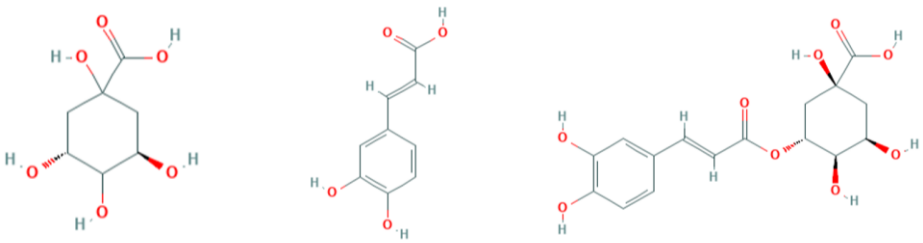

Figure 3.9 – Chemical structure of quinic acid, caffeic acid, and 3-caffeoylquinic acid. Adapted from PubChem.

3.5.3) Volatile compounds identification of the plant by SPME-GC-MS

The volatile profile of the fresh, dried at 70 ºC and lyophilized *S. ramosissima* (R1) was analyzed and the obtained chromatograms are shows in **Figure 3.10**. Due to the goal of applying *S. ramosissima* as an ingredient in foods, the study of volatiles and consequent odours becomes important to evaluate the effects of the drying process on these compounds. The volatile compounds from each processing plant material method were identified and each compound was associated with its calculated linear retention indexes on the DB-5MS column, as well as its relative area (%) (**Appendix E**). In addition, for each compound, the existence of possible odour descriptions was searched in the literature.

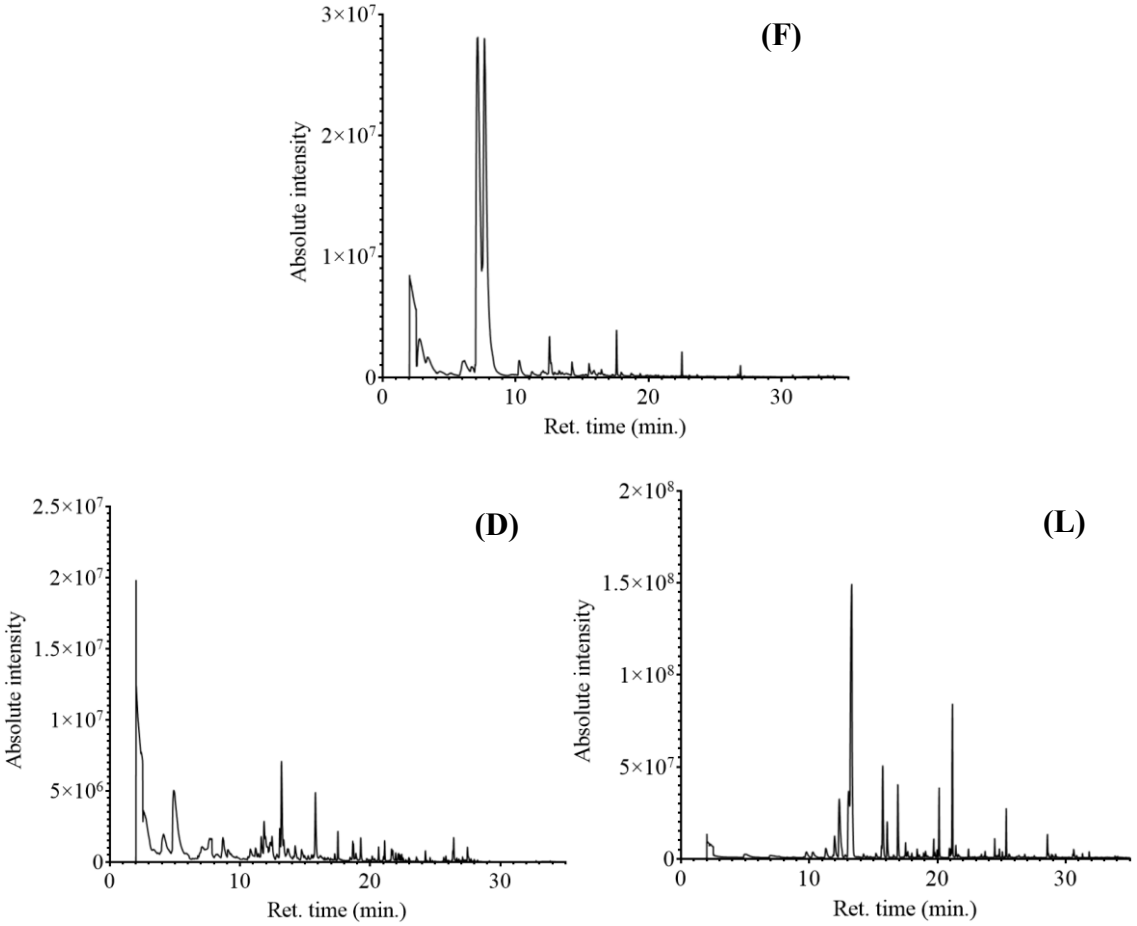

Figure 3.10 – Gas chromatogram of fresh (F), dried at 70 ºC (D) and lyophilized (L) *S. ramosissima* (R1).

As shown in the **Appendix E** table, one hundred volatile compounds were identified in total, of which eighty-four were identified based on the identification attempt by the software library and comparison of the calculated linear retention indexes with the literature. The volatile compounds were also identified based on the spectrum of the target compound and search on PubChem (NCBI).

In the chromatogram of the fresh plant, it is possible to notice two distinct peaks of great intensity, which correspond to (E)-3-hexen-1-ol (47.95%) and 1-hexanol (47.82%). Both alcohols are responsible for green-type odours [143,144]. Compounds such as hexanal (0.26%), 1-methoxy-2-hexene (0.46%), ethyl tiglate (1.51%), (Z)-3-hexen-1-ol acetate (0.33%) and methyl and ethyl benzoate (0.54% and 0.21%, respectively), responsible for odours such as green, herbal, fruity, and floral, were also identified in the fresh *S. ramosissima*.

Regarding the two plant material processing methods used, it is noticed that the chromatogram profiles of the volatile compounds of the dried plant and the lyophilized plant are quite different from each other, especially with regard to the absolute intensities. In the dried halophyte plant chromatogram, compounds such as hexanal (34.16%), (E)-3-hexen-1-ol (1.80%), 2-methylbutanoic acid (7.84%), heptanal (5.14%), 1-octen-3-ol (3.00%), 6-methyl-5-hepten-2-one (3.92%), *p*-cymene (3.08%), limonene (10.18%), 3,4-dimethylcyclohexanol (7.31%) and *β*-cyclocitral (1.97%) caused high intensity peaks. It is noticed that hexanal, an aldehyde that is responsible for herbal, green and grassy odours [143,144], is the most intense verified peak in the dried *S. ramosissima* chromatogram. However, compounds such as 2-methylbutanoic acid, heptanal and 1-octen-3-ol with considerable relative area percentages are observed, and these compounds are described, in the literature, as responsible for some off-odours, like sour, penetrating oily and mushroom-like, respectively [144–146]. In contrast, the lyophilized plant chromatogram detects compounds as *β*-myrcene (2.26%), *α*-phellandrene (8.45%), *p*-cymene (8.80%), 1,8-cineole (38.73%), *β*-thujone (7.21%), *α*-thujone (2.31%), camphor (4.80%) and isobornyl acetate (8.17%), highlighting the large percentage of the relative area of the 1,8-cineole, a terpenoid [147]. These compounds are all responsible for odours that are described as herbaceous, fresh, and green [147–151]. In addition, (Z)-6-nonen-1-ol, the only compound described as odour of seaweed, was identified in the lyophilized *S. ramosissima* (0.30%).

Compounds with higher area percentages in the fresh plant ((E)-3-hexen-1-ol and 1-hexanol) were not detected in the dried nor lyophilized *S. ramosissima* chromatograms, with the exception of 1.80% of (E)-3-hexen-1-ol in the dried halophyte plant chromatogram. In addition, compounds that were detected in the chromatograms of the three different states of plant material, always showed a higher relative area percentage in the dried and lyophilized samples than the fresh halophyte plant, such as hexanal (34.16, 1.30, and 0.26%, respectively), *p*-cymene (3.08, 8.80, and 0.02, respectively), 3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate (0.19, 0.27, and 0.06, respectively), and others.

As seen, both drying methods demonstrate changes in the identified volatile profile, when compared to the fresh halophyte plant. The difference in the volatile profile in the dried plant at 70 ºC plant has been justified by the effect of the temperature and the consequent loss of certain compounds [152]. The lyophilized plant manages to maintain a profile of

main compounds that have characteristic odours identical to the original plant in the fresh state, as described previously. Studies have shown that the lyophilization process, with regard to the volatile profile, is a less aggressive process than drying at high temperatures, managing to maintain a profile more identical to the food in its original state [153].

3.6) Bioactivity

3.6.1) Antioxidant activity

The antioxidant capacity of dried and lyophilized *S. ramosissima* extracts were evaluated according to ORAC and HOSC methods. The values for both methods are expressed in dried plant material (µmol TEAC/g dw) and are shown in **Figure 3.11**.

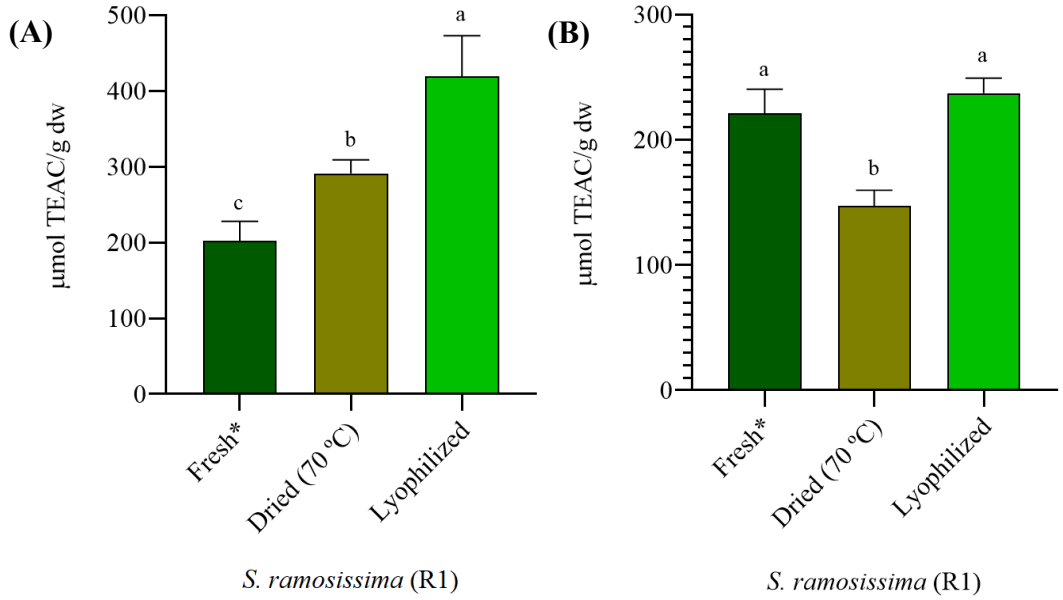

Figure 3.11 – Antioxidant activity assays ORAC (A) and HOSC (B) for fresh, dried at 70 ºC, and lyophilized *S. ramosissima* (R1). Each column represents mean ± standard deviation of triplicate extractions measured in triplicate. The letters (a-c) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.
* The values were converted from fresh weight to dried weight, according to the moisture value.

Taking into consideration the TPC values obtained for both dried extracts, and the previously established positive relationship between the total phenolic content and the antioxidant activity demonstrated, the antioxidant capacity of the lyophilized *S. ramosissima* extract in both ORAC and HOSC assays (418.7 and 237.2 µmol TEAC/g dw, respectively) was expected to be higher than by the dried plant extract (291.1 and

147.2 µmol TEAC/g dw, respectively). It is observed that the temperature of 70 ºC used in the conventional drying of the halophyte plant has negative effects on the antioxidant activity demonstrated. Considering the lower TPC value obtained for the dried at 70 ºC *S. ramosissima* and now the also lower ORAC and HOSC values, when compared to the lyophilized sample, it can be concluded that the temperature used in conventional drying does not allow the preservation of the phenolic compounds from the fresh halophyte plant, occurring oxidation and decomposition of these bioactive compounds, such as quercetin derivatives and chlorogenic acid derivatives [139,141]. This effect has also been observed in other foods such as coffee, a well-known food rich in phenolic compounds namely chlorogenic acid isomers, where with the increase in roasting intensity, there was a greater destruction of phenolic compounds that may not even be compensated by the appearance and formation of other compounds [154].

A significantly higher value is also observed in the lyophilized plant in the ORAC assay when compared to the fresh plant (202.1 µmol TEAC/g dw), and a significantly identical value in the HOSC assay (220.8 µmol TEAC/g dw). Considering the highest concentrations observed in relation to the previously quantified compounds, and the higher TPC, ORAC, and HOSC values, the lyophilization, involving freeze-drying and vacuum, seems to be able to preserve and concentrate these bioactive compounds responsible for the antioxidant capacity of the plant more easily than the conventional drying process.

3.6.2) Antihypertensive activity

The antihypertensive activity evaluated through the ACE inhibition assay was also performed for both extracts of dried and lyophilized *S. ramosissima* and the IC_{50} values, calculated from the different concentration (0-100 mg/ml) curves and respective inhibition percentages, are shown in **Figure 3.12**. The results are expressed in mg/ml.

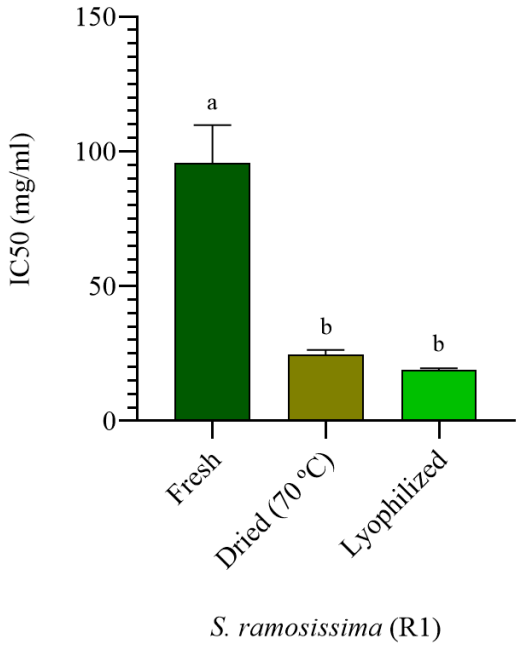

Figure 3.12 – ACE inhibitory assay IC_{50} results, in mg/ml, for the two different *S. ramosissima* (R1) material processing methods (dried and lyophilized) and fresh plant. The letter (a) corresponds to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test.

It is observed that there is no significant difference between the IC_{50} values of the dried at 70 ºC and lyophilized halophyte plant extracts (24.6, and 18.9 mg/ml, respectively). The presence of quercetin-hexosides and caffeoylquinic acid derivatives and their antihypertensive activities reported in the literature [65,66,129] justifies the values obtained of antihypertensive activity for the two types of *S. ramosissima* material. The IC_{50} value of the fresh *S. ramosissima* extract (95.6 mg/ml) is presented again, and as expected, considering that the conversion to dried weight is not possible, demonstrates an antihypertensive activity worse than the other two extracts.

Considering the previously quantified compounds, however, significantly lower concentrations for quercetin-rhamnosyl-hexoside, quercetin-malonyhexoside, and 3,5-dicaffeoylquinic acid were verified in the dried halophyte plant extract when compared to the lyophilized extract. These lower concentrations of phenolic compounds that demonstrate antihypertensive activity in the literature, as previously described, may be the main reason for the slightly higher IC_{50}, and consequently, lower antihypertensive activity verified in the dried extract. Synergistic effects between quercetin and caffeoylquinic activities may also be related to the slightly differences in the antihypertensive activities observed.

Part 3: Consumer acceptance of the inclusion of halophyte plants in the diet as promising ingredients - Sensory analysis with two different foods using dried *S. ramosissima* as an ingredient

Considering the statistically insignificant difference in the antihypertensive activity between dried and lyophilized *S. ramosissima,* the dried halophyte plant was selected to be applied as an ingredient in different foods. The known disadvantages of lyophilization, mainly on an industrial scale, were also taken into consideration, where cost and complexity can be a problem [155]. Due to the easy application of oven-drying method and its lower cost, the dried halophyte plant was selected to be used as an ingredient in popcorn and ketchup, and sensory tests were carried out.

3.7) Sensory analysis

3.7.1) Popcorn test

In the first sensory test, people were asked to evaluate the appearance and the flavour of the sweet and salty popcorn samples with fresh (sample F1) and dried *S. ramosissima* (sample D1). It was possible to perform the test to thirty-one volunteers that were consumers of this product. The means for each attribute are shown in **Figure 3.13**.

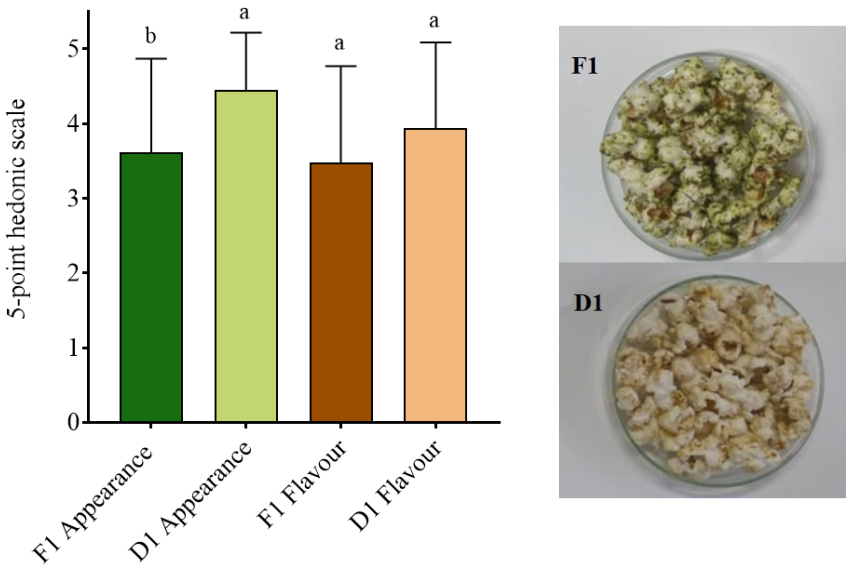

Figure 3.13 – Average evaluation scores for the different attributes of the two popcorns with fresh (F1) and dried *S. ramosissima* (D1) (n = 31). The appearance and flavour were evaluated using a five-point hedonic scale where 1 = "disliked extremely" and 5 = "like extremely". The letters (a and b) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) by unpaired t test. The samples are shown again on the right.

It is observed that the participants generally preferred the D1 sample, which contains dried *S. ramosissima*, with a higher mean in both attributes. It is also possible to see that, although there is no significant difference when comparing the flavour of both samples, the same does not happen in appearance. There is a significant difference between the evaluation of the appearance of both samples, meaning that the bright green colour observed in the sample characteristic of the fresh *S. ramosissima* is not pleasant for the participants. However, the use of dried *S. ramosissima* was successful when compared to the use of fresh halophyte plant. In addition, some consumers commented that the sample with fresh *S. ramosissima* had a "strange texture", while the sample with dried halophyte plant was "crunchier" and did not have an "off taste" as the popcorn with fresh halophyte plant had.

It was also asked if the participant would be interested in buying the products if they saw it for sale on the market, for which the responses obtained were mostly positive for both samples: 19.4% answered "wouldn't buy", 19.4% "would maybe buy", and 61.2% "would buy" for the sample F1; 6.4% answered "wouldn't buy", 9.7% "would maybe buy", and 83.9% "would buy" for the sample D1.

This test concluded that the use of fresh *S. ramosissima* does not become a viable option, given the obtained results, but perhaps dried *S. ramosissima* becomes an interesting ingredient to use as a substitute for table salt. However, the comparison of this sample with sweet and salty popcorn with table salt, with the same sodium content as the amount of halophyte plant used, needs to be evaluated in the future.

It was possible to perform a visual only sensory test at the "ITQB Open Day", where participants were asked to evaluate the appearance of three samples: two sweet and salty popcorn with different amounts of fresh *S. ramosissima* (samples F1 and F2) and one with dried *S. ramosissima* (sample D1). Two hundred and nineteen people, involving a wide range of ages (3-72), including mostly people outside the institute, were able perform the test. People were asked to evaluate the appearance of the three samples and the results are shown in **Figure 3.14**.

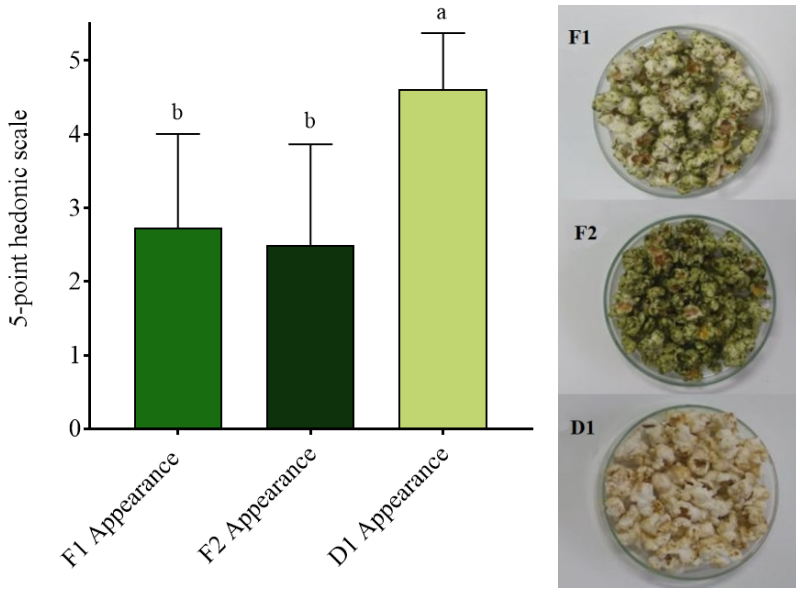

Figure 3.14 – Average evaluation scores for the appearance attribute of the three popcorns with fresh (F1 and F2) and dried *S. ramosissima* (D1) among "ITQB Open Day" participants (n = 219). The appearance was evaluated using a five-point hedonic scale where 1 = "disliked extremely" and 5 = "liked extremely". The letters (a and b) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) according to one-way ANOVA for multiple comparisons by Tukey's test. The samples are shown again on the right.

A preference was observed for the visual aspect of the sample D1 sample, which contains dried *S. ramosissima*. This same sample demonstrated a significant difference in relation to the other two samples with fresh *S. ramosissima*. However, there was no significant difference between the results of the samples with different amounts of fresh *S. ramosissima*. Therefore, once again, a greater preference was observed for the appearance of popcorn with the dried halophyte plant, which does not present a very different visual aspect to the popcorn usually found on the market. In addition, many of the participants were children, where the reaction to the first two "greener" samples with fresh *S. ramosissima* was mostly repulsive due to the "weird colour".

The participants were also asked if they would be interested in buying the products, where the answers obtained were mostly positive for the sample D1, with dried *S. ramosissima* (4.6% answered "wouldn't buy", 5.9% "would maybe buy", and 89.5% "would buy"). The same was not verified when the possible purchase intention was questioned in relation to samples F1 (43.8% answered "wouldn't buy", 17.8% "would maybe buy", and 38.4% "would buy") and F2 (47.5% answered "wouldn't buy", 20.5% "would maybe buy", and 32.0% "would buy"), with fresh *S. ramosissima*.

With these two sensory tests, it was possible to conclude that, despite people appreciating the popcorn samples with both dried and fresh *S. ramosissima*, the bright green appearance that using fresh halophyte gives to popcorn is not yet accepted and appreciated by the population. This means that the insertion of halophyte plants in food, although welcome, is better done in such a way that neither the flavour nor the general appearance of food is altered. The use of dried halophyte plants is perhaps the best option if the goal is to use halophyte plants as an ingredient in food in the future.

3.7.2) Ketchup test

The test with ketchup formulated with dried *S. ramosissima* at 2.2% (sample 2.2%DS) and 3.0% (sample 3.0%DS) test was performed with one hundred and two people, comprising ages between 20 and 59 years old. The information obtained in the questionnaires about each participant is shown in **Table 3.7**. It can be observed that the consumption of ketchup, although it exists, is not so common in Portugal, in which people tend to consume ketchup only rarely or else "once a month". It was also asked what kind of food they usually consume with ketchup, and most responses referred fries and hamburgers. The answers obtained enabled to conclude that each participant was familiar with the conventional product available on the market.

Table 3.7 – Information from participants in the sensory evaluation of ketchups.

	Sensory Test (n = 102)	
	n	%
Gender		
Male	32	31.4
Female	70	68.6
Ages*		
[11-20]	2	2.00
[21-30]	51	51.0
[31-40]	26	26.0
[41-50]	10	10.0
[51-60]	11	11.0
Nationality*		
Portugal	95	94.1
Other	6	5.90
Education*		
High-school or less	11	11.0
Bachelor's degree	21	21.0
Master's degree	38	38.0
Doctor's degree	30	30.0

	Sensory Test (n = 102)	
	n	%
Ketchup consumption frequency		
Daily	0	0.00
Twice a week	3	2.94
Once a week	24	23.5
Once a month	35	34.3
Rarely	39	38.2
Never	1	0.980
Typical foods eaten with ketchup**		
French fries	92	35.5
Pork or other meat sandwiches	39	15.1
Other sandwiches	6	2.32
Pizza	13	5.02
Salad	2	0.772
Pasta	15	5.79
Hamburgers	80	30.9
Other	12	4.63

* Not all participants answered this question, so the percentages were adjusted to the total number of responses.

** The participant was allowed to select more than one option in this question, so the percentages were adjusted to the total number of responses.

People were asked to evaluate the different attributes (appearance, aroma, flavour, and overall impression), for the two samples. The means for each attribute are shown in **Figure 3.15**.

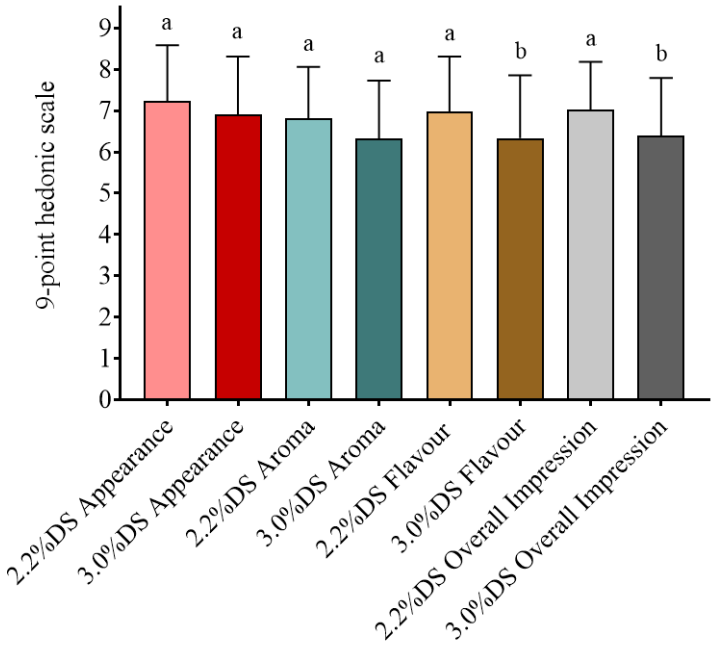

Figure 3.15 – Average evaluation scores for the different attributes of the two ketchups with dried *S. ramosissima* (DS) in 2.2 % and 3.0 % among consumers (n = 102). Each attribute was evaluated using a nine-point hedonic scale where 1 = "disliked extremely" and 9 = "liked extremely". The letters (a and b) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0.05$) between each sample by unpaired t test.

Results presented in **Figure 3.15** show that, for all attributes, the participants preferred the sample 2.2%DS, corresponding to the one with 2.2% dried *S. ramosissima*. However, there is only a significant difference in the attributes of flavour and overall impression. It can be concluded that people preferred ketchup with less addition of dried *S. ramosissima* and, consequently, the product with the lowest salt content. Although it cannot be concluded that the preference was based on the salt content, some consumers commented on the existence of a "slightly strange aftertaste" after consuming the sample 3.0%DS. In addition to the participant's preference for the ketchup with a lower salt content, that sample also contains less than half of the salt content present in several conventional ketchups found on the market (**Appendix F**).

Most people (59.8%) preferred the sample 2.2%DS and, regarding the questions about the possible purchase intention of the preferred product, slightly more than half of the participants responded positively: 53.0% answered "would buy", 43.1% "would maybe buy", and 3.9% "wouldn't buy". However, when asked if they would be willing to pay for that same product twice the price of a conventional ketchup found on the market, it was possible to observe that there was a slightly higher percentage of negative responses (52% of participants answered "no" and 48% "yes"). Given the responses obtained in the frequency of ketchup consumption of the participants, it can be concluded that many consumers are not willing to pay much more than the usual price of a normal sauce. Although the consumption of ketchup is mostly associated with unhealthy eating, the exploration of creating new formulations of tomato-based sauces has been explored [156], and the addition of dried *S. ramosissima* as a table salt substitute becomes an interesting ingredient to also be considered.

4) CONCLUSIONS

This work allowed the study of the most representative halophyte plants species available for consumption in Portugal: *Crithmum maritimum*, *Inula crithmoides*, *Sarcocornia fruticosa* and *Salicornia ramosissima*. Results showed that these plants are rich in proteins, low in fat, and their content in minerals that have been explored as alternatives to the use of sodium in foods, such as calcium, magnesium, and potassium, is interesting. The presence of these minerals and the high content of polyunsaturated fatty acids, namely omega-3, are important factors to consider when using these plants as a possible solution to the problem of hypertension. Furthermore, *C. maritimum*, *I. crithmoides* and *S. fruticosa* can be considered as sources of fiber.

Concerning phytochemicals, such as phenolic compounds, these halophyte plants showed to be rich in phenolic acids such as derivatives of caffeoylquinic and *p*-coumaric acids, as well as flavonoids like quercetin and its derivatives. The presence of these phenolic compounds and their known antioxidant and antihypertensive activities already described in the literature may justify the values obtained for the antioxidant (ORAC and HOSC) and ACE inhibitory assays.

S. ramosissima, which was produced in a natural environment, showed the highest TPC value and antioxidant activity and one of the highest antihypertensive activity values, when compared to *S. ramosissima* produced by hydroponics. The extreme conditions that occur in a natural environment influence the plant's secondary metabolites composition, such as phenolic compounds and, consequently, the values obtained for the bioactivities associated to these compounds.

As these plants may be used in the food industry, it seemed important to evaluate the impact of drying in their composition and bioactivity, as a way to obtain a product adequate for use for a longer period of time. *S. ramosissima* was selected and the impact of drying at 70 ºC and lyophilization was studied. Results showed that the lyophilized *S. ramosissima* presented a higher TPC value, antioxidant activity, and the content of some phenolic compounds such as 3,5-dicaffeoylquinic acid, quercetin-rhamnosyl-hexoside and quercetin-malonyhexoside was also increased. According to the literature, the presence of caffeoylquinic acid and quercetin derivatives in the extracts of fresh, dried, and lyophilized plant justify the obtained antihypertensive activity.

As these halophyte plants can be used as food ingredients, it was important to study the volatile composition, namely the presence of compounds that may be responsible for characteristic odors, and the effect of drying on that composition was studied. Results showed that the lyophilized *S. ramosissima* contained higher peaks corresponding to volatile compounds with aromas that refer to fresh and green notes, similar to the plant in its fresh state. However, the dried plant had a considerable percentage of peaks corresponding to compounds that are described as having unpleasant odours.

Dried *S. ramosissima* was used as an alternative ingredient to salt, in sweet and salty popcorn and ketchup. The results of the sensory tests carried out with the popcorn samples allowed to conclude that the appearance is a key factor to consider when inserting halophyte plants in food, as there was a preference for the sample with dried *S. ramosissima* than with fresh *S. ramosissima*, this later with a characteristic green color. For the ketchup tests, there was a preference for the sample with a lower content of dried *S. ramosissima*, although it has a much lower salt content than that normally used in ketchup sauces.

The results obtained in this work demonstrate that halophyte plants are interesting matrices to explore not only as foods with health benefits but also as functional ingredients to replace the use of conventional salt. The scientific information obtained in this work is considered to be an important contribution that can be used by producers of halophyte plants, in order to help them make their choices concerning the most interesting halophyte plants to produce, commercialize, and valorize.

5) FUTURE PERSPECTIVES

The conclusions presented throughout this preliminary work open doors to other studies relating halophyte plants and future perspectives as ingredients to use in food industry.

It is mandatory to perform more sensory tests in order to conclude about consumers preferences for products with an identical salty taste, but where halophyte plants are used instead of traditional table salt.

More studies to evaluate the power of halophyte plants to lower the incidence of blood pressure are necessary, taking into consideration the presence of phytochemicals. Cellular antioxidant activity tests as well as cytotoxicity tests should be performed.

Moreover, halophyte plant digestive extracts (*in vitro* digestion) should be done to better understand the effect of digestion on the composition of the plant and have an idea about the bioaccessibility and bioavailability of the compounds and its effect on the antioxidant and antihypertensive activities. Additionally, *in vivo* studies to evaluate the real effect of the consumption in a regular diet and their components bioavailability would be an interesting approach in the future.

Portugal produces numerous halophyte plants, which allow a good diversity of species and, consequently, bioactive components. Other halophyte plants of different species have already been acquired and preliminary studies (TPC, ORAC and HOSC assays) have already started to be done (**Appendix G**), in order to contribute to a better knowledge about these plants, based in scientific criteria and evaluate which are the most promising ones, considering their sensory properties, composition, and, consequently, their bioactivity.

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APPENDIXES

Appendix A – Nutritional parameters, fatty acids profile, and mineral composition of the produced ketchup samples 2.2%DS and 3.0%DS with dried *Salicornia ramosissima*, used in the sensory tests.

	2.2%DS	**3.0%DS**
Nutritional profile (g/100g)		
Moisture	71.3 ± 0.71a	68.4 ± 0.68b
Proteins	1.68 ± 0.07b	2.0 ± 0.08a
Total fat	0.20 ± 0.004a	0.20 ± 0.004a
Ashes	1.86 ± 0.07b	2.34 ± 0.09a
Total dietary fiber	2.20 ± 0.07b	2.70 ± 0.08a
Carbohydrates	22.8 ± 0.91a	24.4 ± 0.98a
Energy (kcal/100g)	104 ± 4.16a	113 ± 4.51a
Salt	0.91 ± 0.12b	1.38 ± 0.18a
Fatty acids profile (%)		
Myristic acid (C14:0)	2,62 ± 0.01a	2,45 ± 0.01b
Palmitic acid (C16:0)	24,0 ± 0.01a	24,0 ± 0.01a
Stearic acid (C18:0)	3,83 ± 0.01a	3,73 ± 0.01b
Oleic acid (C18:1)	5,70 ± 0.01a	5,65 ± 0.01b
Linoleic acid (C18:2)	40,9 ± 0.01a	40,5 ± 0.01b
Linolenic acid (C18:3)	19,2 ± 0.01b	20,2 ± 0.01a
Arachidic acid (C20:0)	0,84 ± 0.01a	0,86 ± 0.01a
Arachidonic acid (C20:4)	0,38 ± 0.01a	0,24 ± 0.01b
Behenic acid (C22:0)	0,74 ± 0.01b	0,82 ± 0.01a
Lignoceric acid (C24:0)	0,76 ± 0.01b	0,87 ± 0.01a
Σ SFA	33,5 ± 0.01a	33,0 ± 0.01b
Σ MUFA	6,10 ± 0.01a	6,02 ± 0.01b
Σ PUFA	60,5 ± 0.01b	61,0 ± 0.01a
PUFA/SFA	1.81 ± 0.01b	1.85 ± 0.01a
Mineral composition (mg/100g)		
Sodium (Na)	364 ± 47.3b	550 ± 71.5a
Calcium (Ca)	21.4 ± 2.57a	27.3 ± 3.28a
Potassium (K)	330 ± 69.3a	370 ± 77.7a
Iron (Fe)	1.47 ± 0.21a	1.34 ± 0.19a
Magnesium (Mg)	39.9 ± 5.59a	50.4 ± 7.06a
Manganese (Mn)	0.28 ± 0.04a	0.35 ± 0.05a
Zinc (Zn)	0.22 ± 0.03a	0.28 ± 0.04a
Copper (Cu)	nd	0.1 ± 0.009a

nd – not detected (limit of quantification = 0.05 g/100g), SFA – total saturated fatty acids, MUFA – total monounsaturated fatty acids, PUFA – total polyunsaturated fatty acids. Results are presented as mean ± SD. In each row, the letters (a-b) mean statistically significant differences ($p < 0.05$) by unpaired t test.

Appendix B - Tables of LC-DAD-ESI-MS/MS phytochemical compounds identification of the halophyte plants.

Peak (Fig. 8)	t_r (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (% base peak)	Tentative identification	References
Crithmum maritimum						
-	7.35	301	191	[191]: 111(50), 87(35), 85(100)	citric acid	[103]
1	8.05	262	133	[133]: 133(30), 115(30), 71(100)	malic acid	[105]
2	9.6	258	191	[191]: 173(80), 111(5), 87(10), 85(100)	quinic acid	[103,105]
-	30.8	285	315	[315]: 153(30), 109(100)	protocatechuic acid-glucoside	[157,158]
6	36.78	283	325	[325]: 163(100), 119(95)	*p*-coumaric acid-glucoside	[35,107,108]
7	37.62	296,327	353	[353]: 191(100)	5-caffeoylquinic acid	[114]
-	39.62	290	355	[355]: 271(15), 253(100), 209(30), 181(25), 107(40)	pinobanksin-3-pentanoate	[121]
-	40.92	270,330	593	[593]: 593(100), 341(5), 311(10)	apigenin 6-glucoside-7-glucoside	[159]
9	43.15	311	337	[337]: 191(100), 173(15), 163(15), 119(10), 111(10), 93(35)	*p*-coumaroylquinic acid (isomer 1)	[109]
11	45.13	307	337	[337]: 191(100), 163(5)	*p*-coumaroylquinic acid (isomer 2)	[109]
-	46.73	255,352	609	[609]: 609(100), 429(30), 301(5), 300(100)	quercetin-3-rutinoside	[121,160]
12	47.78	255,355	463	[463]: 463(50), 301(55), 300(100)	quercetin-3-hexoside	[35,44]
-	49.25	265,325	431	[431]: 413(5), 341(15), 312(100), 311(20)	apigenin 6-glucoside	[159]
16	49.77	268,297,329	515	[515]: 353(40), 330(100), 191(70), 179(25)	3,5-dicaffeoylquinic acid	[35,105]
-	50.2	270,286,334	607	[607]: 607(20), 299(100)	diosmetin-7-rutinoside	[161]
-	51.9	285	475	[475]: 163(100)	*p*-coumaric acid derivative	[35,107]
Inula crithmoides						
	7.33	301	215	[215]: 191(10), 179(15)	quinic acid derivative	[103,105]
1	8.5	271	133	[133]: 133(5), 115(20), 71(100)	malic acid	[105]
2	9.32	269	191	[191]: 171(20), 155(2), 111(60), 87(100), 85(50)	quinic acid	[103,105]
-	9.85	261	243	-	ni	-
-	12.15	257	-	-	ni	-

Peak (Fig. 8)	t_r (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (% base peak)	Tentative identification	References
-	14.63	274	238	-	ni	-
-	26.55	289	315	[315]: 300(15), 151(25)	isorhamnetin	[162]
-	28.17	281	407	[407]: 407(60), 244(30), 169(10), 165(20)	gallic acid derivative	[163]
-	29.72	259,298	197	[197]: 197(65), 182(60), 153(25)	syringic acid	[103]
-	30.5	282	315	[315]: 300(40), 151(40)	rhamnetin	[162]
-	31.78	256,296	259	[259]: 179(60), 146(100), 135(5)	caffeic acid derivative	[44,164,165]
-	32.97	283	465	-	ni	-
-	33.97	275	349	-	ni	-
-	34.8	281	305	[305]: 225(40), 97(95)	gallocatechin	[166]
-	35.57	274	341	[341]: 179(100), 135(75)	caffeic acid-glucoside	[44,164,165]
5	36.18	276	163	[163]: 119(15), 95(100)	*p*-coumaric acid	[35,107]
7	37.02	298,322	353	[353]: 191(100)	5-caffeoylquinic acid	[114]
-	38.23	284	455	[455]: 455(80), 169(40), 125(20)	gallic acid derivative	[163]
-	39.03	280	411	[411]: 411(100), 169(15), 125(95)	gallic acid derivative	[163]
8	40.42	280	327	[327]: 285(40), 267(10), 239(20), 180(20), 165(30), 139(50)	pinobanksin-5-methyl ether-3-acetate	[121]
10	44.65	284,324	367	[367]: 247(55), 193(15), 191(100)	feruloylquinic acid	[167]
-	46.05	280,315	357	-	ni	-
-	46.87	280	419	[305]: 305(80), 225(10), 97(50)	gallocatechin derivative	[166]
-	47.73	284	369	[369]: 369(10), 255(10), 193(40), 179(15), 165(100), 107(30)	piscidic acid derivative	[168]
-	49.23	284	395	[395]: 395(100), 163(10), 119(20)	*p*-coumaric acid derivative	[35,107]
-	50.98	280,325	431	[431]: 431(20), 334(10), 210(15), 181(35), 147(40), 121(100)	cinnamic acid derivative	[169]
18	52.47	281,324	517	[517]: 397(15), 355(15), 179(35), 135(35)	caffeic acid-glucuronide-glucoside (isomer 2)	[124]
Sarcocornia fruticosa						
-	7.42	301	215	[215]: 191(10), 179(15)	quinic acid derivative	[103,105]
1	8.58	265	133	[133]: 115(15), 71(80)	malic acid	[105]

Peak (Fig. 8)	t_r (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (% base peak)	Tentative identification	References
2	9.43	271	191	[191]: 173(20), 171(20), 127(20), 111(55), 87(100), 85(55)	quinic acid	[103,105]
-	9.9	263	279	-	ni	-
3	18.72	275	261	[261]: 181(20), 163(15), 135(10), 119(5), 97(100)	*p*-coumaric acid derivative	[35,107]
-	27.8	275	343	[343]: 191(10)	5-galloylquinic acid	[170]
4	29.78	281,328	353	[353]: 191(65), 179(80), 135(30)	3-caffeoylquinic acid	[113]
-	32.7	281	285	-	ni	-
-	33.48	307	259	[259]: 179(65), 135(100)	caffeic acid derivative	[44,164]
-	34.9	278,318	305	[305]: 305(10), 225(25), 97(100)	gallocatechin	[166]
-	36.2	265	321	-	ni	-
7	37.13	296,326	353	[353]: 191(100), 179(10), 173(10)	5-caffeoylquinic acid	[44,114]
-	39.15	275	387	[387]: 387(85), 163(20), 119(65)	*p*-coumaric acid derivative	[35,107]
-	40.4	-	455	-	ursolic acid	[171,172]
-	41.05	268	209	-	ni	-
-	41.6	270,335	303	[303]: 303(5), 97(100)	dihydroquercetin	[173]
9	42.85	254,353	337	[337]: 337(20), 191(95), 173(40), 163(25)	*p*-coumaroylquinic acid (isomer 1)	[109]
-	44.78	280,320	499	[499]: 337(40), 163(10), 111(5), 93(5)	3-*p*-coumaroyl-5-caffeoylquinic acid	[174]
-	46.58	314	269	-	ni	-
-	47.25	274,320	437	[437]: 437(40), 289(20)	epicatechin-pentose	[175]
-	47.93	255,265,350	609	[609]: 609(100), 477(30), 459(10), 315(60), 299(30), 165(25)	rhamnetin hexosyl pentoside	[176]
15	49.07	254,266,352	623	[623]: 623(100), 477 (10), 487(25), 315(35), 215(10), 214(40)	isorhamnetin 3-robinobioside	[123]
-	50.08	307	429	[429]: 391(100), 337(40), 173(80), 163(15), 119(20)	*p*-coumaric acid derivative	[35,107]
-	50.92	278,319	335	[335]: 335(90), 163(100)	*p*-coumaric acid derivative	[35,107]
-	51.37	279,32	561	[561]: 561(100), 337(50), 163(60), 119(40)	*p*-coumaric acid derivative	[35,107]
***Salicornia ramosissima* (R1)**						
-	7.12	301	215	[215]: 191(10), 179(15)	quinic acid derivative	[103,105]
1	8.57	273	133	[133]: 133(65), 115(55), 113(100), 71(30)	malic acid	[105]
2	9.38	263	191	[191]: 111(80), 87(100), 85(45)	quinic acid	[103,105]

Peak (Fig. 8)	t_r (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (% base peak)	Tentative identification	References
4	29.53	301,325	353	[353]: 191(100), 179(80), 135(20)	3-caffeoylquinic acid	[113]
-	32.77	280,317	285	[285]: 153(25), 152(100), 108(75), 109(20)	protocatechuic-acid-arabinoside	[103,177]
-	34.48	278	305	[305]: 305(10), 225(30), 97(100), 59(80)	gallocatechin	[166]
-	35.35	284,318	355	[355]: 137(80), 93(100)	salicylic acid derivative	[178]
5	36.03	274	163	[163]: 119(100)	*p*-coumaric acid	[35,107]
7	36.93	300,327	353	[353]: 191(100)	5-caffeoylquinic acid	[44,114]
-	38.93	267,345	193	[193]: 134(100), 161(30), 178(10)	ferulic acid	[103]
-	40.23	269,332	355	[355]: 193(100), 178(20), 161(40), 134(10)	ferulic acid-glucoside	[103]
-	41.27	268,337	303	[303]: 303(5), 97(100)	dihydroquercetin	[173]
9	42.68	274,312	337	[337]: 191(100), 173(15), 163(55)	*p*-coumaroylquinic acid (isomer 1)	[109]
-	44.07		371	[371]: 249(15), 121(80), 113(10)	saccharide	[179]
-	45.37	256,336	319	-	ni	-
-	46.48	268,336	609	[609]: 301(100), 151(50)	quercetin-rhamnosyl-hexoside	[176]
-	47.1	274,335	519	[519]: 315(100), 301(45), 300(5), 299(35)	quercetin-methyl-ether derivative (isomer 1)	[180]
12	47.78	255,352	463	[463]: 463(35), 301(65), 300(100)	quercetin 3-hexoside	[35,44]
14	48.77	251,358	549	[549]: 505(100), 463(15), 301(25), 300(50)	quercetin-malonyhexoside	[113,181]
16	49.5	302,332	515	[515]: 353(100), 325(40), 191(75), 179(45)	3,5-dicaffeoylquinic acid	[35,105]
17	50.52	302,329	515	[515]: 479(3), 353(100), 191(15), 179(35), 173(50)	4,5-dicaffeoylquinic acid	[35,105]
18	51.25	266,331	517	[517]: 397(100), 355(75), 179(40), 135(10)	caffeic acid-glucuronide-glucoside (isomer 2)	[124]
-	51.78	256,268,346	519	[519]: 519(20), 350(50), 315(100), 300(5)	quercetin-methyl-ether derivative (isomer 2)	[180]
-	52.33	328	563	[563]: 503(20), 473(15), 459(70), 443(5), 383(5), 353(2)	apigenin-6-arabinosyl-8-glucoside (isoschaftoside)	[122]
-	59.92	287	953	[953]: 953(100), 767(50), 575(50), 285(15)	kaempferol derivative	[182]
***Salicornia ramosissima* (R2)**						
-	7.33	301	215	[215]: 191(10), 179(15)	quinic acid derivative	[103,105]
1	8.58	265	133	[133]: 133(65), 115(55), 113(100), 71(30)	malic acid	[105]

Peak (Fig. 8)	t_r (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (% base peak)	Tentative identification	References
2	9.75	261	191	[191]: 111(100), 87(60), 85(75)	quinic acid	[103,105]
-	14.7	273		-	ni	-
4	29.65	296,325	353	[353]: 191(100), 179(85), 135(60)	3-caffeoylquinic acid	[113]
-	32.92	317	285	[285]: 153(30), 152(100), 108(100), 109(20)	protocatechuic-acid-arabinoside	[103,177]
-	34.22	280,310	305	[305]: 225(30), 97(100), 59(75)	gallocatechin	[166]
5	36.12	274	163	[163]: 119(100)	*p*-coumaric acid	[35,107]
7	37.02	296,326	353	[353]: 191(100), 179(10), 173(20)	5-caffeoylquinic acid	[44,114]
-	39.07	284,318	193	[193]: 134(10), 161(50), 178(5)	ferulic acid	[103]
-	40.23	269,330	355	[355]: 193(30), 178(15), 161(20), 134(10)	ferulic acid-glucoside	[103]
-	41.97	278,320	259	-	ni	-
9	42.73	288,312	337	[337]: 191(100), 173(20), 163(25)	*p*-coumaroylquinic acid (isomer 1)	[109]
-	44.22		371	[371]: 249(45), 121(100), 113(40)	saccharide	[179]
11	44.77	302	337	[337]: 191(100), 179(20), 173(10), 163(5)	*p*-coumaroylquinic acid (isomer 2)	[109]
-	47.2	282,316	519	-	ni	-
13	48.5	296,326	515	[515]: 353(100), 335(20), 191(25), 179(55), 173(80)	3,4-dicaffeoylquinic acid	[35,105]
16	49.62	296,326	515	[515]: 353(100), 191(85), 179(55)	3,5-dicaffeoylquinic acid	[35,105]
17	50.62	294,326	515	[515]: 353(100), 191(15), 179(55), 173(80)	4,5-dicaffeoylquinic acid	[35,105]
-	52.48	284,322	955	[955]: 955(100), 477(5)	isorhamnetin-3-glucoside	[183]

t_r – retention time, ni – not identified

Appendix C - Table of phytochemical compounds identified by LC-DAD-ESI-MS/MS in fresh (F), dried at 70 ºC (D) and lyophilized (L) *Salicornia ramosissima* (R1).

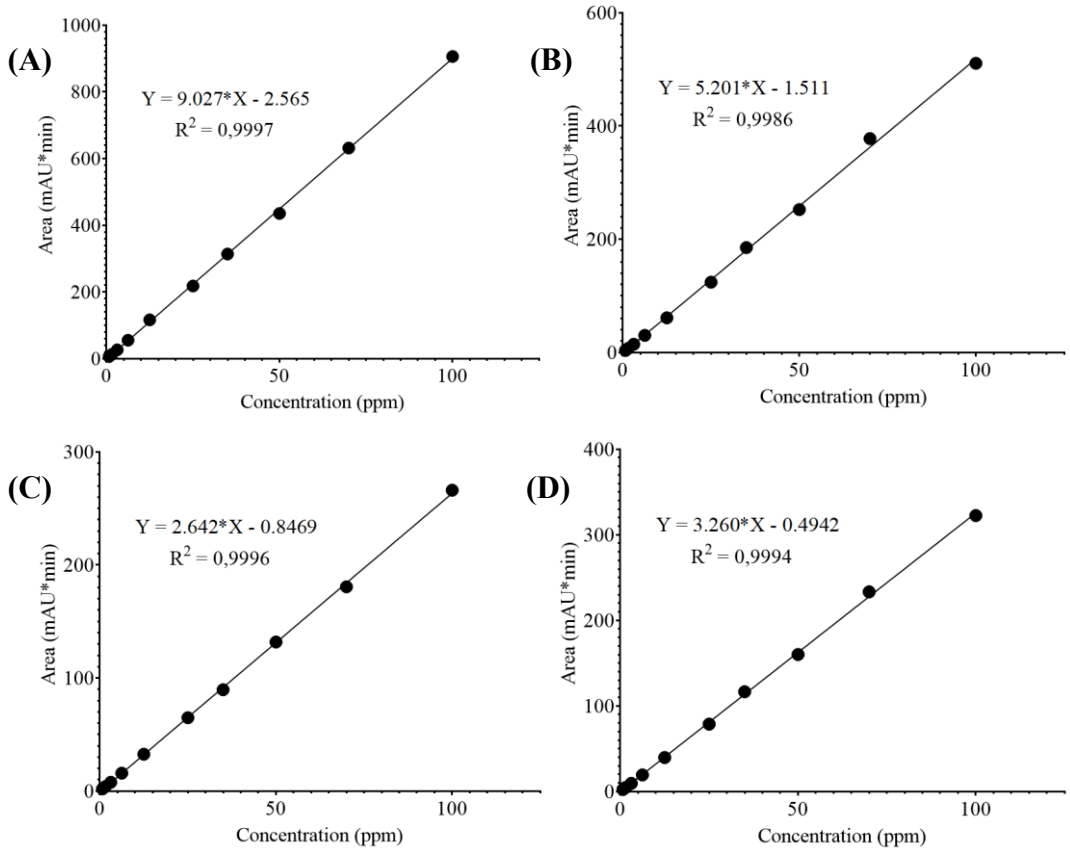
Peak (Fig. 13)	t_r (min)			λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (*)	Tentative identification	References
	F	D	L					
-	7.12	-	7.15	301	215	191, 179	quinic acid derivative	[103,105]
-	8.57	8.8	8.38	275	133	133, **115**, 113**,** 71	malic acid	[105]
1	9.38	9.42	9.28	260	191	**111**, 87, 85	quinic acid	[103,105]
2	29.53	28.03	29.53	300,325	353	**191**, **179**, 135	3-caffeoylquinic acid	[113]
-	32.77	-	32.77	280,317	285	153, **152**, 108, 109	protocatechuic-acid-arabinoside	[103,177]
-	34.48	32.62	34.4	275	305	305, 225, **97**, 59	gallocatechin	[166]
-	35.35	34.35	-	284,318	355	137, **93**	salicylic acid derivative	[178]
-	-	-	35.38	281,332	355	273, 253, 191, 173	hydrocaffeoylquinic acid	[44]
-	36.03	34.88	35.98	274	163	**119**	*p*-coumaric acid	[35,107]
3	36.93	35.88	36.92	300,327	353	**191**	5-caffeoylquinic acid	[44,114]
-	38.93	38.03	38.92	267,345	193	**134**, 161, 178	ferulic acid	[103]
-	40.23	-	40.28	269,332	355	**193**, 178, 161, 134	ferulic acid-glucoside	[103]
-	41.27	39.55	41.25	268,337	303	303, **97**	dihydroquercetin	[173]
-	42.68	41.85	42.68	274,312	337	**191**, 173, **163**	*p*-coumaroylquinic acid (isomer 1)	[109]
-	44.07	43.48	44.08		371	249, **121**, 113	saccharide	[179]
-	-	44.6	-	330	337	215, **191**, 173, 163	*p*-coumaroylquinic acid (isomer 2)	[109]
-	45.37	-	45.6	256,336	319	**295**, 294, 187, 97	ni	-
4	46.48	46.07	46.48	270,340	609	**301**, 151	quercetin-rhamnosyl-hexoside	[176]
-	-	46.5	-	300	449	**253**, 118	*p*-coumaric acid benzyl ester derivative	[184]
-	47.1	-	-	274,335	519	315, **301**, 300, 299	quercetin-methyl-ether derivative (isomer 1)	[180]
5	47.78	47.35	47.78	255,352	463	463, **301**, 300	quercetin 3-hexoside	[35,44]
-	-	-	48.18	284,329	517	517, **355**, **179**, 135	caffeic acid-glucuronide-glucoside (isomer 1)	[124]

Peak (Fig. 13)	t_r (min)			λ_{max} (nm)	$[M-H]^-$ *m/z*	LC–DAD–ESI-MS/MS *m/z* (*)	Tentative identification	References
	F	D	L					
6	48.77	48.35	48.75	251,358	549	**505**, 463, **301**, 300	quercetin-malonyhexoside	[113,181]
7	49.5	49.08	49.53	302,332	515	**353**, 325, **191**, **179**	3,5-dicaffeoylquinic acid	[35,105]
8	50.52	50.13	50.5	302,329	515	479, **353**, 191, 179, **173**	4,5-dicaffeoylquinic acid	[35,105]
-	51.25	50.6	50.97	266,331	517	**355**, **179**, 135	caffeic acid-glucuronide-glucoside (isomer 2)	[124]
-	51.78	51.42	51.77	256,268,346	519	519, 350, **315**, 300	quercetin-methyl-ether derivative (isomer 2)	[180]
-	52.33	-	-	328	563	503, 473, **459**, 443, 383, 353	apigenin-6-arabinosyl-8-glucoside (isoschaftoside)	[122]
-	59.92	-	59.88	287	953	**953**, **767**, **575**, 285	kaempferol derivative	[182]

***** The values of *m/z* in bold are the ions that have relative intensities greater than 50% in the three extracts.

t_r – retention time, ni – not identified.

Appendix D – Standard curves of gallic acid (A), 3-caffeoylquinic acid (B), quercetin 3-hexoside (C), and quercetin-acetylhexoside (D) used to quantify compounds in the halophyte plants.



Appendix E - Table of volatile compounds identified in fresh (F), dried at 70 ºC (D) and lyophilized (L) *Salicornia ramosissima* (R1).

	t_r (min.)					Calc. LRI			Lit.		Area%		
Number	**F**	**D**	**L**	**Compound name**	**Odour description**	**F**	**D**	**L**	**LRI**	**References**	**F**	**D**	**L**
1	5.117	4.920	4.998	hexanal	herbal, grassy, green	803	798	799	801	[185,186]	0.26	34.16	1.30
2	6.700	-	-	1-methoxy-2-hexene	not found	847	-	-	-	PubChem CID: 5366215	0.46	-	-
3	-	-	6.962	(E)-2-hexenal	floral, herbal	-	-	853	850	[186,187]	-	-	0.68
4	7.138	7.067	-	(E)-3-hexen-1-ol	green	858	855	-	852	[143,188]	47.95	1.80	-
5	-	-	7.635	3-methyl-1-pentanol	fruity, floral	-	-	871	852	[189,190]	-	-	0.04
6	7.653	-	-	1-hexanol	woody, sweet, green, fruity	867	-	-	872	[144,187]	47.82	-	-
7	-	7.684	-	2-methylbutanoic acid	cheesy, sour	-	872	-	876	[145]	-	7.84	-
8	-	8.685	8.758	heptanal	penetrating oily, harsh	-	900	902	902	[144,185,191]	-	5.14	0.10
9	-	9.079	-	butyrolactone	cheesy, burnt sugar	-	911	-	915	[192,193]	-	1.29	-
10	-	9.278	-	2-3-dimethylpyrazine	nutty, cocoa-like	-	916	-	918	[144,194]	-	0.17	-
11	-	-	9.569	*α*-thujene	herbal, green, weak earthy	-	-	924	929	[150,195]	-	-	0.05
12	-	-	9.786	*α*-pinene	oily, green	-	-	930	933	[148]	-	-	0.88
13	10.260	-	-	ethyl tiglate	fruity	943	-	-	939	[196]	1.51	-	-
14	-	-	10.274	camphene	sweet	-	-	943	947	[148,197]	-	-	1.09
15	-	10.616	-	*γ*-valerolactone	sweet, herbaceous	-	953	-	950	[198,199]	-	0.25	-
16	-	10.792	-	benzaldehyde	hazelnut, roasty	-	958	-	962	[200,201]	-	1.39	-
17	-	-	11.288	*β*-pinene	woody, green, pine-like	-	-	971	979	[197,202]	-	-	1.37
18	-	11.416		3,5,5-trimethyl-2-hexene	not found	-	975	-	977	[203]	-	0.31	-
19	-	11.627	11.646	1-octen-3-ol	mushroom	-	981	981	980	[146,195]	-	3.00	0.01
20	-	11.848	11.848	6-methyl-5-hepten-2-one	banana-like	-	987	987	988	[188,204]	-	3.92	0.05
21	12.055	11.953	-	2-pentylfuran	floral, fruit	992	990	-	990	[205,206]	0.07	1.14	-
22	-	-	11.973	*β*-myrcene	herbaceous, sweet	-	-	990	991	[148]	-	-	2.26
23	12.233	12.094	-	hexanoic acid	unpleasant, rancing, metallic	997	993	-	993	[207,208]	0.07	0.60	-
24	-	12.321	-	decane	gasoline-like, fishy	-	1000	-	1000	[209,210]	-	0.99	-

	t_r (min.)					Calc. LRI			Lit.		Area%		
Number	**F**	**D**	**L**	**Compound name**	**Odour description**	**F**	**D**	**L**	**LRI**	**References**	**F**	**D**	**L**
25	-	-	12.329	*α*-phellandrene	fresh, green	-	-	1000	1008	[149,197]	-	-	8.45
26	-	12.372	-	octanal	fruity, green, citrus	-	1001	-	1005	[203,211]	-	0.16	-
27	12.663	-	-	(Z)-3-hexen-1-ol acetate	fruity, floral	1010	-	-	1007	[186,187]	0.33	-	-
28	-	-	12.793	*α*-terpinene	resinous	-	-	1014	1017	[149,212]	-	-	0.24
29	-	12.834	-	2-methylpentyl formate	not found		1015	-	-	PubChem CID: 537217	-	0.39	-
30	12.917	-	-	hexyl acetate	fruit, herb	1017	-	-	1019	[190,213]	0.11	-	-
31	13.125	13.049	13.074	*p*-cymene	green, fruity, aromatic	1024	1021	1022	1027	[148,202]	0.02	3.08	8.80
32	13.267	13.183	-	limonene	pine/chemical, floral/fresh	1028	1025	-	1029	[146,200,214]	0.16	10.18	-
33	-	-	13.306	1,8-cineole	eucalyptus, spicy, pepper	-	-	1029	1032	[147,200,214]	-	-	38.73
34	-	13.353	-	2-ethyl-1-hexanol	green, flowery, green cucumber	-	1031	-	1029	[208,215]	-	1.14	-
35	13.517	-	-	2-hexenoic acid	not found	1035	-	-	-	PubChem CID: 5282707	0.08	-	-
36	-	13.643	-	3-octen-2-one	rose	-	1039	-	1040	[206,216]	-	0.29	-
37	-	13.719	-	phenylacetaldehyde	lilac, flora	-	1041	-	1043	[145,200]	-	0.16	-
38	13.808	-	-	benzyl alcohol	floral, fruity, rose	1044	-	-	1045	[217]	0.06	-	-
39	-	-	14.225	gamma-terpinene	green, woody	-	-	1057	1059	[148]	-	-	0.29
40	-	-	14.502	*trans*-sabinene hydrate	spicy, weak fruity	-	-	1065	1060	[150]	-	-	0.13
41	-	14.721	14.741	3,5-octadien-2-one	plastic	-	1071	1072	1098	[146]	-	1.19	0.04
42	-	-	15.194	*α*-terpinolene	fresh, green	-	-	1086	1086	[149,218]	-	-	0.37
43	-	15.503	-	2-(4,5-Dimethyl-1-cyclopenten-1-yl)-2-propanol	not found	-	1095	-	-	PubChem CID: 91691661	-	0.30	-
44	15.508	-	-	methyl benzoate	eucalyptus, phenolic, wood, medicinal	1095	-	-	1094	[219,220]	0.54	-	-
45	-	-	15.633	linalool	pleasant scent, floral	-	-	1099	1097	[187,202]	-	-	0.48
46	-	15.640	-	2,6,11-trimethyldodecane	not found	-	-	1099	1102	[221]	-	0.14	-
47	-	-	15.724	*β*-thujone	camphoraceous-herbal	-	-	1102	1119	[150]	-	-	7.21

	t_r (min.)					Calc. LRI			Lit.		Area%		
Number	**F**	**D**	**L**	**Compound name**	**Odour description**	**F**	**D**	**L**	**LRI**	**References**	**F**	**D**	**L**
48	-	15.789	-	3,4-dimethylcyclohexanol	not found	-	1104	-	1103	[222]	-	7.31	-
49	-	-	16.069	*α*-thujone	warm-herbal, minty	-	-	1113	1114	[150,223]	-	-	2.31
50		16.165	-	farnesol	floral, sweet, lily-like,	-	1116	-	-	PubChem CID: 3327	-	0.09	-
51	16.233	-	-	phenylethyl Alcohol	sweet, perfume, floral, bee wax	1118	-	-	1117	[188]	0.11	-	-
52	-	-	16.566	1,3,8-p-menthatriene	green, cucumber, floral	-	-	1129	1130	[146,189]	-	-	0.14
53	-	-	16.903	camphor	camphoraceous, fresh	-	-	1140	1131	[150]	-	-	4.80
54	-	-	17.429	*trans*-pinocamphone	cedar, camphoreous, woody	-	-	1157	1157	[224,225]; PubChem CID: 11038	-	-	0.08
55	-	-	17.627	borneol	camphoraceous, earthy	-	-	1163	1165	[150,226]	-	-	0.18
56	-	-	17.674	myrcenol	citrus, floral, fresh	-	-	1165	-	PubChem CID: 10975	-	-	0.27
57	-	-	17.847	*cis*-pinocamphone	cedar, camphoreous, woody	-	-	1171	1172	[225]; PubChem CID: 11038	-	-	0.04
58	17.925	-	-	ethyl benzoate	flowery	1173	-	-	1172	[200,227]	0.21	-	-
59	-	-	17.983	terpinen-4-ol	green, fruity, citrus-like	-	-	1175	1178	[148]	-	-	0.21
60	-	18.469	18.403	*α*-terpineol	minty, fresh vegetable, green	-	1191	1189	1189	[208,228]	-	0.32	0.43
61	-	-	18.566	*cis*-dihydrocarvone	cooling, fresh, minty	-	-	1194	1193	[229,230]	-	-	0.11
62	-	18.659	-	safranal	saffron-like	-	1197	-	1197	[231,232]	-	1.16	-
63	18.785	18.713	-	dodecane	alkane-like, chemical	1201	1200	-	1200	[203,233]	0.02	1.02	-
64	-	18.892	-	decanal	soapy, chemical	-	1205	-	1205	[200,234]	-	0.42	-
65	-	-	18.910	verbenone	sweet, floral, camphor-like	-	-	1206	1204	[192,235]	-	-	0.25
66	-	-	19.056	4,7-dimethyl-benzofuran	smoke, moss, spicy	-	-	1211	1220	[146,209]	-	-	0.34
67	-	-	19.223	cuminaldehyde	sweet, fresh	-	-	1217	1207	[148,236]	-	-	0.07
68	19.358	19.270	-	*β*-cyclocitral	sweet-tobacco, grape	1221	1218	-	1220	[200,237]	0.08	1.97	-
69	-	-	19.697	thymol methyl ether	oregano-like, smoky-like, woody	-	-	1233	1233	[195,238]	-	-	0.93
70	-	-	19.782	octyl-acetate	fruity, herbal	-	-	1236	1222	[190,239]	-	-	0.22

	t_r (min.)					Calc. LRI			Lit.		Area%		
Number	F	D	L	Compound name	Odour description	F	D	L	LRI	References	F	D	L
71	-	-	19.957	isothymol methyl ether	burnt, smoky-like, woody	-	-	1242	1244	[240]; PubChem CID: 161716	-	-	0.31
72	-	20.150	-	2',4',6'-trimethylacetophenone	not found	-	1249	-	-	PubChem CID: 15461	-	0.39	-
73	-	-	20.116	1,2-diisopropylbenzene	sharp, penetrating	-	1248	-	-	PubChem CID: 11345	-	-	3.29
74	21.083	21.019	-	4,6-dimethyldodecane	not found	1281	1280	-	1285	[241]	0.01	0.16	-
75	-	-	21.037	(Z)-6-nonen-1-ol	fresh, seaweed, green, cucumber, fatty	-	-	1280	-	PubChem CID: 5362792	-	-	0.30
76	-	-	21.157	isobornyl acetate	herb, woody, sweet, minty	-	-	1284	1285	[151,226]	-	-	8.17
77	-	-	21.417	thymol	thyme-like, spicy	-	-	1294	1293	[150,238]	-	-	0.56
78	-	21.639	21.600	2-undecanol	tallowy, soapy	-	1301	1300	1287	[234,242]	-	0.97	0.10
79	-	21.730	-	2,4-diethyl-1-heptanol	not found	-	1305	-	1321	[243]	-	0.64	-
80	-	21.964	-	2-isopropyl-5-methyl-1-heptanol	not found	-	1314	-	1300	[244]; PubChem CID: 545941	-	0.59	-
81	-	22.149	-	2,2,4,4,6,8,8-heptamethylnonane	not found	-	1321	-	1321	PubChem CID: 20414	-	0.46	-
82	-	22.965	-	1-hydroxy-2,4,4-trimethyl-3-pentanyl 2-methylpropanoate	fruity	-	1351	-	-	[245]; PubChem CID: 156477	-	0.39	-
83	-	-	23.416	(3-Isopropenyl-2-methylcyclopentyl)methyl acetate	not found	-	-	1368	-	PubChem CID: 539231	-	-	0.14
84	23.633	23.553	23.699	3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	apple, fresh, cucumber	1375	1373	1379	1376	[246,247]	0.06	0.19	0.27
85	-	24.238	-	tetradecane	alkane-like, chemical	-	1399	-	1413	[233,248]	-	0.75	-
86	-	-	24.451	(4E)-4-tridecen-1-yl acetate	not found	-	-	1407	-	PubChem CID: 5365748	-	-	0.78

	t_r (min.)					Calc. LRI			Lit.		Area%		
Number	**F**	**D**	**L**	**Compound name**	**Odour description**	**F**	**D**	**L**	**LRI**	**References**	**F**	**D**	**L**
87	-	-	24.730	*trans*-caryophyllene	fresh, fruity	-	-	1418	1419	[149,228]	-	-	0.11
88	-	-	24.813	thymohydroquinone dimethyl ether	earthy, moldy	-	-	1422	1426	[249,250]	-	-	0.37
89	-	-	25.048	2-acetoxytetradecane	not found	-	-	1431	-	PubChem CID: 536524	-	-	0.24
90	-	-	25.228	alloaromadendrene	woody	-	-	1438	1442	[251,252]	-	-	0.05
91	-	-	25.349	2-allyl-1,4-dimethoxy-3-methyl-benzene	not found	-	-	1443	-	PubChem CID: 606035	-	-	2.10
92	-	25.645	25.561	nerylacetone	rose, fresh, green, magnolia	-	1455	1452	1452	[253,254]	-	0.24	0.15
93	-	25.810	-	octadecane	fuel-like	-	1462	-	-	PubChem CID: 11635	-	0.24	-
94	-	26.350	-	4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	not found	-	1483	-	1483	[255]	-	0.52	-
95	-	26.412	-	*β*-ionone	dry fruit, floral	-	1486	-	1486	[145]	-	1.49	-
96	-	26.644	-	10-methylnonadecane	not found	-	1495	-	-	PubChem CID: 530070	-	0.22	-
97	26.692	-	-	valencene	fruity, flowery	1496	-	-	1487	[256,257]	0.06	-	-
98	-	27.094	-	2,4-bis(2-methyl-2-propanyl)phenol	phenolic	-	1513	-	1513	[258,259]	-	0.19	-
99	-	27.470	-	dihydroactinidiolide	coumarin-like, musky	-	1529	-	1528	[260,261]	-	1.26	-
100	-	-	30.835	1-heptadecene	earthy, moss	-		1677	1673	[201,262]	-	-	0.11

t_r – retention time, LRI – linear retention index.

Appendix F – Nutritional table of two different ketchups found on the market.

	Valores por 100 g Values for 100 g
Energia Energy	496 kJ 117 kcal
Lipidos / Fat	< 0,5 g
- dos quais saturados/ - of which saturates/	0 g
Hidratos de carbono/ Carbohydrates /	27 g
- dos quais açúcares - of which sugars	22 g
Proteína / Protein	1,4 g
Sal / Salt	2,0 g

Declaração nutricional	Por 100 g	Por porção**	%* por porção**
Energia	381 kJ/90 kcal	59 kJ/14 kcal	< 1%
Lipidos	< 0,5 g	< 0,5 g	< 1%
dos quais saturados	< 0,1 g	< 0,1 g	< 1%
Hidratos de carbono	20 g	3,0 g	1%
dos quais açúcares	19 g	2,8 g	3%
Proteínas	1,4 g	< 0,5 g	1%
Sal	1,8 g	0,27 g	5%

*% Dose de referência para um adulto médio (8400 kJ/2000 kcal) ** 1 porção = 15g
Esta embalagem contém aproximadamente 18 porções.

Appendix G – TPC (A), expressed in mg GAE/g fw, and antioxidant activities by ORAC (B) and HOSC (C), expressed in µmol TEAC/g fw, of numerous other halophyte species. Besides Horta da Ria (HR) and RiaFresh (RA), plants were also bought from Salina Greens (SG; Setúbal, Portugal) and tested. Wild *C. maritimum* was also collected from rocks (Rock) and dunes (Dune) and tested. The letters (a-e) correspond to the statistical analysis performed to calculate the existence of a significant difference ($p < 0,05$) according to one-way ANOVA for multiple comparisons by Tukey's test.