Universidade de Aveiro Departamento de Química
Ano 2017

Carla Sofia Barros Martins

Caracterização química e avaliação biológica da *Salicornia ramosissima*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, especialização Biotecnologia Alimentar, realizada sob a orientação científica da Doutora Sílvia M. Rocha, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

‘’ Families are the compass that guides us. They are the inspiration to reach great heights, and our comfort when we occasionally falter.’’

- Brad Henry

Aos meus pais e irmãos.

o júri

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palavras-chave

salicórnia (*Salicornia ramosissima* J.Woods), compostos bioativos, compostos lipofílicos, compostos fenólicos, minerais, atividade antioxidante, atividade anti-inflamatória

Resumo

Atualmente, existe um elevado interesse na valorização de recursos naturais como fontes de compostos bioativos com potenciais efeitos benéficos para a saúde. A salicórnia é uma planta halófita que tem sido usada na alimentação e na medicina tradicional e mais recentemente no desenvolvimento de novos produtos alimentares, ilustrando o interesse da sua caracterização e avaliação. Esta planta encontra-se dispersa mundialmente, estando presente em algumas regiões em Portugal, nomeadamente na Ria de Aveiro e na Ria da Formosa, no Algarve. Esta planta cresce espontaneamente em ambientes salinos, estando inserida num ambiente de elevado stress. O género *Salicornia* compreende cerca de 25-30 espécies sendo a *Salicornia ramosissima* uma das menos estudadas atendendo à sua composição química. Alguns compostos bioativos são reportados nesta espécie, nomeadamente ácidos gordos, esteróis e compostos fenólicos, no entanto a informação encontra-se muito dispersa atendendo aos seus efeitos biológicos e composição. Neste sentido, o conhecimento da composição química e dos potenciais efeitos biológicos da *S. ramosissima* é extremamente importante para introduzir novas aplicações. Assim, o objetivo principal desta tese foi a caracterização química e avaliação *in vitro* da atividade antioxidante e anti-inflamatória extratos da *S. ramosissima,* recolhida na ria de Aveiro.

Foram recolhidas quatro amostras, no estado de frutificação, em três locais da ria de Aveiro, estas amostras foram tratadas e armazenadas para posterior caracterização. Inicialmente foi estudado a fração lipofílica (extratos de diclorometano) da planta por GC-qMS, seguida do estudo da fração polar (extratos de metanol seguidos de extração com éter de petróleo/água) por LC-QqQ-MS. Posteriormente foram analisados minerais essenciais por ICP-OES e componentes potencialmente tóxicos por ICP-MS. Na fase final foi avaliada ainda a atividade antioxidante e anti-inflamatória dos extratos da fração polar da planta.

De um total de 35 compostos da fração lipofílica, o ácido linolénico, o ácido linoleico, o stigmasterol, o β-sitosterol e o ácido palmítico foram os compostos maioritários. O conteúdo total de lipofílicos variou entre 541 e 5412 mg/100g peso seco. Na fração polar, o conteúdo em fenóis totais variou entre 1391 e 3398 mg de equivalentes de ácido gálico por 100g. A amostra vermelha da Marinha dos peixinhos (MPR) apresentou o maior conteúdo de fenóis. Da análise detalhada dos compostos fenólicos, foram identificados 32, sendo que 22 são reportados pela primeira vez nesta espécie. Isorhamnetina é composto maioritário presente nesta espécie. MPR apresentou um maior número de compostos identificados assim como um maior conteúdo estimado (1676.6 μg/g de extrato peso seco). O estudo dos minerais revelou que o sódio é o mineral mais abundante em todas as amostras, no entanto o consumo de 5g desta planta fresca numa salada corresponde apenas a 6.0-7.1% da dose diária recomendada (DDR) para este mineral.

Relativamente ao selénio, magnésio e potássio pode contribuir para a DDR dos mesmos com 1.9-2.6%, 1.3-2.1% e 0.2-0.3%, respetivamente. Os estudos *in vitro* da atividade antioxidante foram expressos em valores de EC_{50}. Os diferentes estudos permitiram avaliar o potencial da *S. ramosissima* como fonte de antioxidantes, estando os compostos fenólicos relacionados com esta atividade (r^2>0.77). A atividade anti-inflamatória foi avaliada através da inibição da produção de dois metabolitos do metabolismo do ácido araquidónico (TXA_2 e PGE_2). Apesar de nenhum dos extratos inibir a produção de PGE_2, o extrato da Marinha dos Peixinhos (MP) e o extrato do Rio Boco (BC) inibiram a produção de TXA_2 em 33.2% e 18.1%. A aspirina, conhecida pelos seus efeitos em processos anti-inflamatórios foi usada na mesma metodologia, inibindo o PGE_2 e o TXA_2 em 18% e 69.3%, respetivamente. Sendo que algumas reações neste metabolismo envolvem radicais, os compostos previamente identificados nos extratos podem ser fundamentais para a atividade reportada. Além disso sendo que a aspirina inibe preferencialmente TXA_2, pode-se inferir que os extratos de *S. ramosissima* apresentam um comportamento similar.

Em conclusão os resultados obtidos permitiram a caracterização química sumária da *S. ramosissima* da Ria de Aveiro, com especial destaque para os compostos lipofílicos e fenólicos e ainda a presença de minerais essenciais. A presença de compostos com potenciais efeitos benéficos para a saúde humana (flavonoides, fitoesteróis e ácidos gordos ω-3 e ω-6) pode ser um fator determinante para a valorização desta planta assim como a presença de baixo teor de sal, podendo ser usado como coadjuvante na dieta de forma a diminuir o risco de doenças cardiovasculares.

O efeito anti-inflamatório dos extratos da *S. ramosissima* revelou o seu potencial impacto na produção de TXA_2.

keywords

glasswort (*Salicornia ramosissima* J.Woods), bioactive compounds, lipophilic compounds, phenolic compounds, minerals, antioxidant activity, anti-inflammatory activity

abstract

Nowadays, there is a growing interest on the valuation of natural resources as sources of bioactive compounds with potential health benefits. Glasswort (*Salicornia* spp.) has been used on foods and on folk medicine, and more recently on new food products development, which illustrates the potential and interest to study the characterization and evaluate the potential of this plant. Halophytes are world widely distributed being present in some regions in Portugal, namely in Ria de Aveiro and Ria da Formosa, Algarve. They grow spontaneously in saline environments and they are inserted in a high stress environment. *Salicornia* genus comprises about 25-30 specie, being *S. ramosissima*, one of the species less studied considering its chemical composition. Some bioactive compounds are reported on this species, namely fatty acids, sterols and phenolic compounds. However, the published data shows a dispersed information regarding their biological effects and chemical composition. In this context, the knowledge of the chemical composition and the potential health benefits of *S. ramosissima* from Ria de Aveiro, it is extremely important, contributing to improve the applications for this halophyte. Therefore, the main objective of this thesis was the chemical characterization and the evaluation of *in vitro* antioxidant and anti-inflammatory activities in *S. ramosissima* extracts from Ria de Aveiro lagoon.

Four samples, in fructification stage, were collected at 3 locals from Ria de Aveiro lagoon, being treated and stored for further characterization. Initially, the lipophilic fraction (extracts of dichloromethane) from the plant was studied by GC-qMS, followed by the study of the polar fraction (methanol extracts followed by liquid-liquid extraction with petroleum ether/water) in LC-QqQ-MS. Then, essential minerals were quantified by ICP-OES and toxic compounds were analyzed by ICP-MS. Finally, the antioxidant and anti-inflammatory activity were evaluated on the polar fraction extracts.

From 35 lipophilic compounds, linolenic acid, linoleic acid, stigmasterol, β-sitosterol and palmitic acid were the major compounds present. The total lipophilic content ranged between 541-5412 mg/100g dry weight. In polar fraction, the total phenolic compounds ranged between 1391-3398 mg of acid gallic equivalents per 100g. Marinha dos Peixinhos Red sample (MPR) showed higher phenolic content. From the detailed characterization of polar fraction, 32 compounds were identified, being 22 reported for the first time in this species. Isorhamnetin was the major compound present in this fraction and MPR showed higher amount of detected compounds and higher phenolic estimated content (1676.6 μg/g of extract dry weight). The mineral analysis revels that sodium is the major mineral present in all samples. However, an intake of 5g fresh plant, in a salad, corresponds to a sodium recommended daily intake (RDI) of 6.0-7.1%.

For selenium, magnesium and potassium, the intake of 5g would represent a RDI of 1.9-2.6%, 1.3-2.1% and 0.2-0.3%, respectively. *In vitro* antioxidant activity studies were expressed in IC_{50} values. The different studies allowed to evaluate the potential of *S. ramosissima* as a source of antioxidants, being the phenolic compounds linked to this activity (r^2> 0.77). The *in vitro* anti-inflammatory activity was assessed by testing the inhibition of the production of two arachidonic acid metabolites (TXA_2 and PGE_2). None of the extracts inhibited the PGE_2 production. However, Marinha dos Peixinhos (MP) and Boco River (BC) inhibited TXA_2 production in 33.2% and 18.1%, respectively. Aspirin, known for its effects on anti-inflammatory processes, was used in the same methodology, inhibiting PGE_2 and TXA_2 by 18% and 69.3%, respectively. Since, some reactions in arachidonic acid metabolism involve radicals, the compounds previously identified on the extracts may be fundamental to understand this reported activity. Furthermore, aspirin preferentially inhibits TXA_2, so it can be concluded that the *S. ramosissima* extracts showed a similar behavior.

In conclusion, the results allowed to a knowledge of the general chemical characterization of *S. ramosissima* plant, from Ria de Aveiro lagoon, with emphasis to the lipophilic and phenolic compounds as well as the presence of essential minerals. The presence of compounds with reported health benefits (flavonoids, ω-3 and ω-6 fatty acids and phytosterols), may be a crucial factor on the valuation of *S. ramosissima* as well as the sodium levels. This plant could be used as a salt substitute and a coadjuvant in human diet in civilizational diseases, namely in reducing the risk of cardiovascular diseases. The anti-inflammatory effect of *S. ramosissima* extracts revealed its potential impact on the production of TXA_2.

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Aims and outline of this thesis

Exploitation of natural products with bioactive compounds and biological effects that contributes to health benefits is increasing, since the consumption of some natural matrices as plants may be capable of preventing some diseases, particularly these with growing incidence in last decades (e.g. cardiovascular, cancer and diabetes) (1). In the last decades, the consumers' desire for a health promoting diet through the consumption of food products that naturally have diverse bioactive compounds. They believe that foods contribute directly to their health and the interest in naturalness and clean label is growing Nowadays, the food products are design to satisfy, to provide necessary nutrients for humans and to prevent nutrition-related diseases and improve physical and mental well-being to the consumers. In this regard, there are a growing interest on studying plants that might contribute to the health-promoting lifestyle (2).

Salicornia species is a worldwide distributed halophyte plant and there has been a growing interest in this halophyte due to its health potential as a raw material for a natural substitute of marine salt (3,4). In Portugal, there are two main locations where this halophyte can be founded, Ria de Aveiro lagoon and Ria da Formosa, Algarve (5,6). *Salicornia* species have been used in folk medicine with several applications, such as antioxidant, anti-inflammatory and antidiabetic (7). Besides, the higher consumption of sodium is a worldwide concern that are related to cardiovascular diseases (8). Since these diseases are an increasing problem in the last few decades, development of salt alternatives associated with a source of nutritive and bioactive compounds on diet may reduce the sodium intake and the incidence of these diseases. Furthermore, different parameters, as harvesting season and different locations, that might affect their chemical profile are not enough explored. Thus, the knowledge of chemical composition of this halophyte is extremely important to create new applications, particularly in food industry

The main objective of the present thesis is the chemical characterization of *S. ramosissima* and evaluation of antioxidant and anti-inflammatory activities. The secondary metabolites families that will be take into consideration are sterols, fatty acids and phenolic compounds, since these components are the compounds most studied and documented in this halophyte. Phytosterols are considered biologically active and have an important role in human health because they reduce exogenous cholesterol absorption by displacement in the intestinal lumen. Phenolic compounds are essential to plant physiology, growth, reproduction and exhibit diverse health benefits, such as anti-allergenic, anti-artherogenic, antimicrobial, anti-inflammatory, antioxidant and cardioprotective effects. Furthermore, the essential minerals and the toxic compounds

content will be also analysed. The present master thesis is organized in four chapters according to the sequence illustrated in Figure 1.

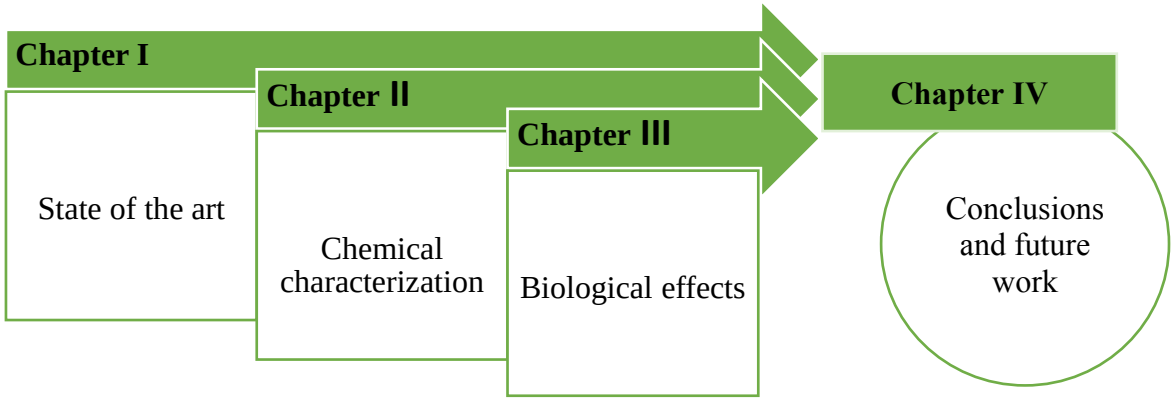

Figure 1- Thesis workflow: from state of the art to chemical characterization and biological effects of *Salicornia ramosissima*.

Chapter I reviews the most relevant literature data from *Salicornia* species with special focus on *S. ramosissima*, namely its taxonomic classification, geographic distribution, detailed review of the chemical composition of glasswort, focusing on lipophilic and phenolic compounds, as well as the minerals content. The potential health benefits of *Salicornia* species, with emphasis on anti-inflammatory and antioxidant activity are also discussed.

Chapter II describes the screening of lipophilic and phenolic compounds as well as mineral content of *S. ramosissima* and discuss this plant as source of bioactive compounds.

Chapter III describes the evaluation of antioxidant and anti-inflammatory activity of *S. ramosissima* extracts. The antioxidant profile was detailed evaluated by performing several tests (ABTS, DPPH, nitric oxide scavenger capacity, ferric redu and lipid peroxidation). Anti-inflammatory activity was performed by testing the effects of *S. ramosissima* extracts on prostaglandin E_2 and thromboxane A_2 production in human leukemic U937 macrophages

Chapter IV highlights the main conclusions of this thesis and future work.

Chapter I – Bibliographic Revision

1.1. *Salicornia* species

1.1.1. Taxonomic Classification and Morphology

The morphology of *Salicornia* has been studied and according to phylogenetic studies this plant is morphologically closely related to *Sarcocornia* (9). These two genus differ from all other Salicornioideae (subfamily) since they both include the presence of a horseshoe-shaped embryo and the absence of perisperm in seeds (10). *Salicornia* differs from *Sarcocornia* in two aspects: (i) *Salicornia* have an annual life versus a perennial life of *Sarcocornia;* (ii) *Salicornia* flowers form a characteristic triangle with a larger central and two smaller lateral flowers, while *Sarcocornia* flowers are arranged in a horizontal row (3,9). *Salicornia* (glasswort, saltwort, samphire) is a genus of annual, apparently leafless halophytic plants (adapted to saline environments), belonging to Chenopodiaceae family, one of the most advanced families of Caryophyllales order (3,11). This genus contains ca. 25 to 30 species (3), being *Salicornia europaea*, *Salicornia bigelovii*, *Salicornia brachiata* and *Salicornia herbacea* the most studied and documented (12).

Regarding the morphology of this plant, *Salicornia* spp. has green, succulent and articulated stems; flowers that consist in 3–4 fused tepals; stamens; an ovary with one ovule; and a style that is apically divided into 2–3 stigmatic lobes (3). However, the high phenotypic plasticity in *Salicornia* genus is responsible for the complexity inside the genus and differences in the morphology are responsible for the taxonomic difficulties (3,11,13). In certain species, distinction based solely on morphology is complicated, that there are descriptions of species aggregates, subspecies and micro species. *Salicornia ramosissima* J. Woods and *Salicornia europa* are, sometimes, named as micro species (3,11,14), including *S. europaea agg*. due to the extreme difficulty that exists in differentiating them, although these species are being genetically distinct (6,13,14).

Salicornia ramosissima J. Woods is a species with no apparent leaf and erect may reach 40 cm (6,11). This halophyte has branches that are approximately straight, inflorescences in spikes terminals comprising two opposed sets of each three flowers. The central flower is rounded, almost circular, and their stems are fleshy (simple and/or highly branched) segmented by joints. In this joints are present trace leaves (11,14–16). The different components present in *S. ramosissima* J. Woods are shown in Figure 2.

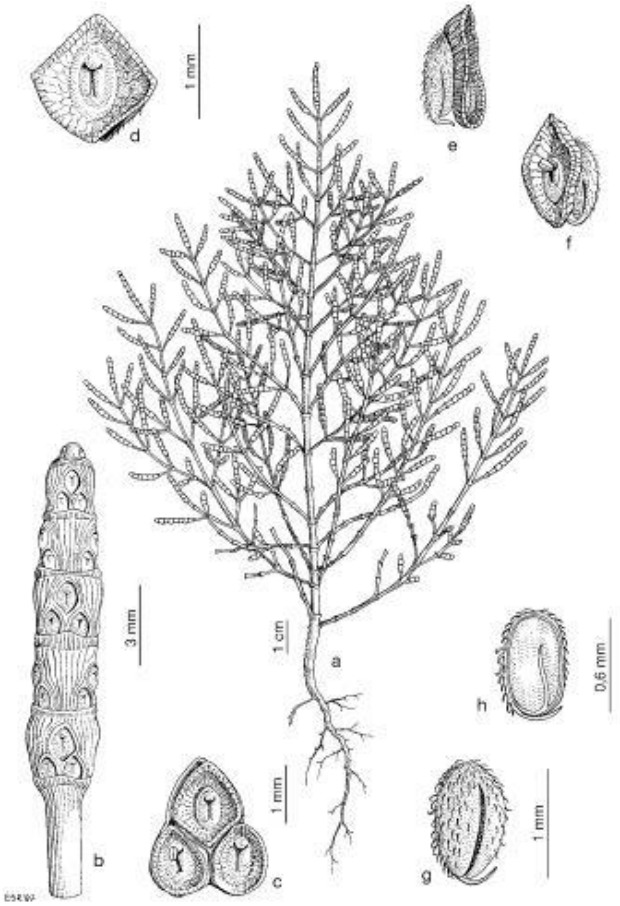

Figure 2 - *Salicornia ramosissima* constituents. a) habit; b) inflorescence; c) floriferous summit; d) flower; e) seed with perianth remains in lateral vision; f) fruitful perianth with stamen; g) seed; h) longitudinal section of seed.

Many species of the genus *Salicornia* are green but their branches are firstly purplish-red in the fall. This happens in *S. ramosissima* in some countries, named as "purple glasswort" in England due to this specific color (Figure 3) (7,11). In Portugal and Spain, this halophyte is known by "green salt".

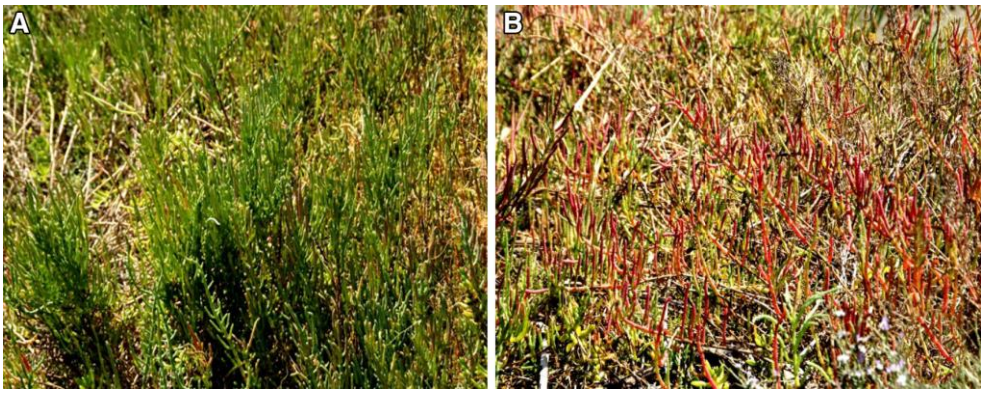

Figure 3 - A - *Salicornia* spp. in spring and summer is green; B - *Salicornia* spp. red and purple in autumn with high salt concentration (12).

The life cycle of *Salicornia* species is characteristically summer-annual, while in subtropical environments, this plant can persevere for more than a year (11). *Salicornia* demonstrates delayed growth until late in the summer (vegetative stage) that tends to begin in May and prolongs to July/August. In late summer, when tides are large and the sea floods the upper marsh, conditions are better and growth rate increases, which is associated with flowering (*Salicornia* produce flowers, usually in August and September, these flowers occur on the spikes – fertile segments in the end of all branches) and seed production (occurs in November – smalls seeds are produced by the two lateral flowers of the cymule and a large seed by the flower in the center of the cymule), being fructification (flowers production) and seed production the other two stages of *Salicornia* (6,17)

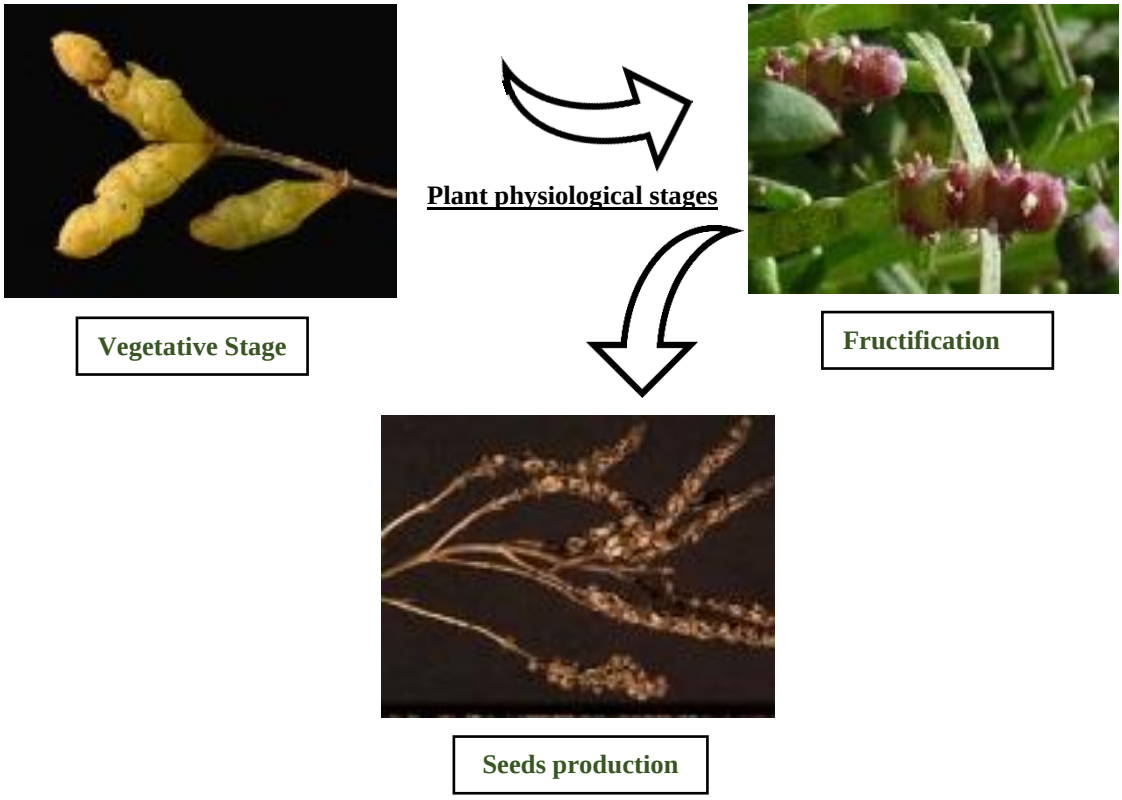

Figure 4 - The three different physiological stages of *Salicornia* species.

1.1.2. Geographic Distribution

Halophytes, cover at the most 2 % of terrestrial plant species, containing a diversity of plants (18). *Salicornia* species are widely dispersed in the world, being present in Europe, Asia, North America and South Africa (12,17,18). *S. europaea, S. obscura, S. prostrata, S. pusilla, S. ramosissima, S. dolichostachya, subsp. Strictissima, S. fragilis and S. nitens* are species distributed in Europe (3,17,18), being some of these species present at British Isles (11). *S. persica, S. perennans and S. bigelovii* are species that can be collected at least in Germany, the Netherlands, England, Israel and Kazakhstan (18). *Salicornia* genus occurs also extensively in temperate and subtropical regions of north hemisphere and South Africa. However, this genus can't be found in South America, Australia (3) and in saline environments of inland North America, more specifically in Rittman, Ohio (19), the case of *S. europaea*. *Salicornia* species can be found in the following countries: Scandinavia, France, Portugal, Spain, Italy, Turkey, Egypt, Bahrain, Kuwait, Oman, Qatar, Saudi Arabia, United Arab Emirates, Yemen, Syria, Iraq, Former U.S.S.R, Siberia and China (3). The case of *S. ramosissima*, this species essentially distributed in West Europe and West Mediterranean (3). The Figure 5 represents the worldwide distribution for all the *Salicornia* species.

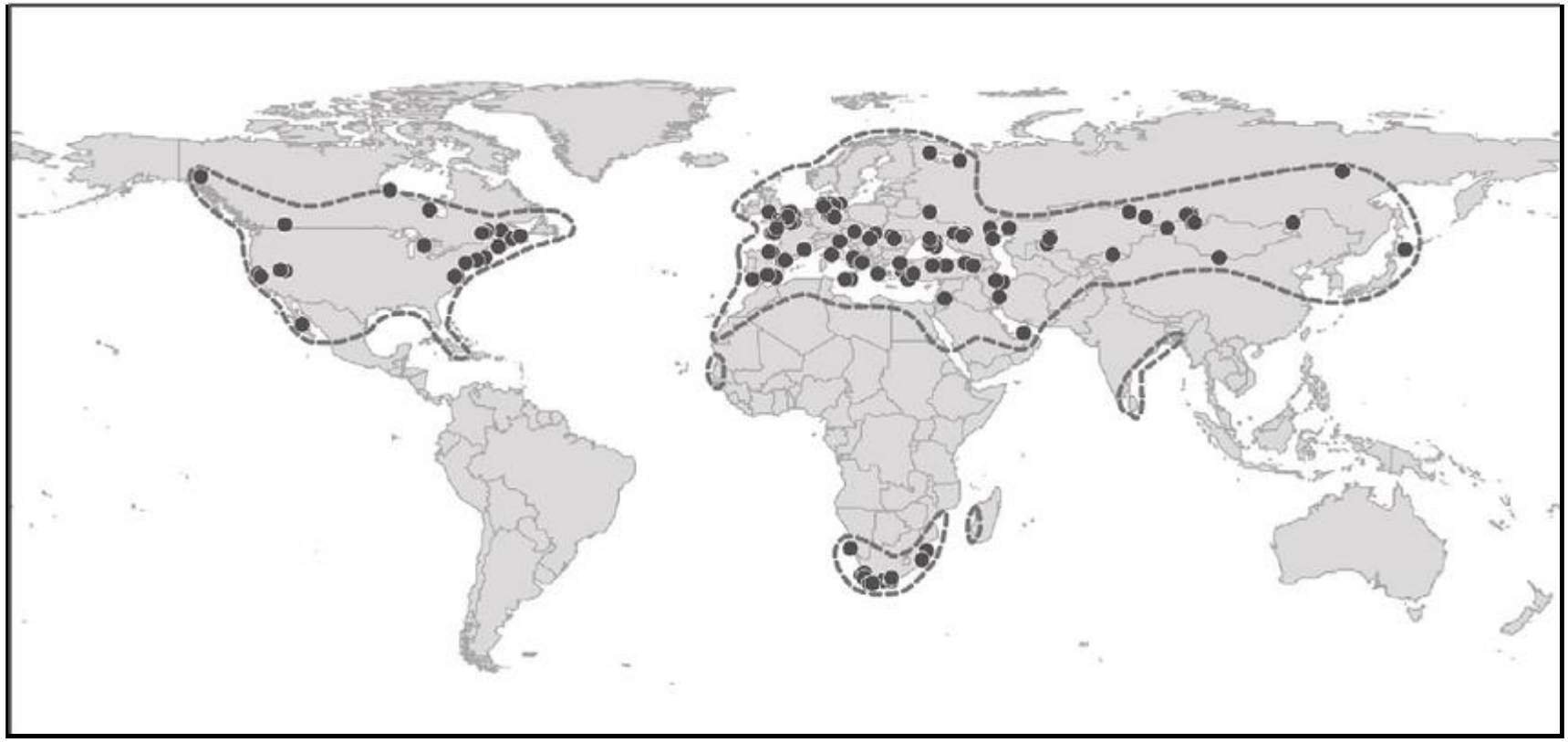

Figure 5- Worldwide distribution of *Salicornia* species (3); ● - *Salicornia* spp distribution; --- continental dispersion (North America, Eurasia and South Africa).

In Portugal, *Salicornia* species are dispersed in all coast, being *S. ramosissima*, the most reported species. In fact, Aveiro and Algarve regions are the regions associated to this halophyte plant and to this specific species (5,6,17,20), however this plant can be found in other regions. The distribution of *S. ramosissima*, in Portugal, it is represented in Figure 6.

Figure 6- Distribution of *S. ramosissima* in Portugal (5,6,21).

Ria de Aveiro lagoon, located on the Northwest Atlantic coast of Portugal, is one of the biggest geographical accidents of the Portuguese coast and it has a very particular geometry with narrow channels and significant intertidal zones, namely mud flats and salt marshes (22). The flora from this region of Portugal includes among others, *Salicornia ramosissima*, *Sarcocornia perennis*, *Halimione portucaloides*, *Tamarix africana*, *Juncus maritimus*, *Spartina maritima* (23,24), being *S. ramosissima* widely distributed in the salt marsh of Ria de Aveiro lagoon, present in low and mid salt marshes of this lagoon and the natural occurrence of this species is preferentially small places not occupied by other halophytes (6).

1.1.3. Chemical composition

This section will give an overview of the general composition of *Salicornia* species, including the cutting edge of *S. ramosissima,* followed by a specific overview of lipophilic and phenolic compounds and minerals composition. The general composition of *Salicornia* species and seeds are synthetized in Table 1.

Table 1- General composition of different *Salicornia* species and seeds.

Species	***S. ramosissima*** (g/100g) dw	***S. herbacea*** **(**g/100g**)** fw	***S. herbacea*** (g/100g) dw	***S. bigelovii*** *Seeds* (g/100g) dw	***S. bigelovii*** (g/100g) fw
Moisture	84.5 ± 0.2[1]	73.9[2]	____	____	88.42 ±1.36[5]
Crude protein	5.20 ±0.29[1]	2.0[2]	____	33.07 ± 1.67[4]	1.54 ± 0.10[5]
Crude lipid	____	0.3[2]	____	____	0.37 ± 0.01[5]
Fat	1.87 ± 0.18[1]	____	1.70 ± 0.15[3]	____	____
Ash	____	6.1[2]	8.01 ± 0.36[3]	6.06 ± 1.10[4]	4.36 ± 0.37[5]
Salt	____	3.9[2]	____	____	____
Carbohydrates	____	____	____	____	4.48 ± 0.46[5]
Sugar	____	13.4[2]	____	____	____
Crude fiber	____	____	____	5.79 ± 1.50[4]	0.83 ± 0.13[5]
Amino acids	____	1.525[2]	____	____	1.086[5]
Vitamin C	____	____	____	____	0.00584[5]

– not reported; fw – fresh weight; dw – dry weigh
[1]Reference (5) [2]Reference (25) [3]Reference (26) [4]Reference (27) [5]Reference (28)

It was also reported that *S. bigelovii* contains vitamins A and C, minerals (mostly magnesium, iron and calcium) and essential amino acids, making a suitable and potential candidate as a nutritional dietary supplement. Their protein levels (2.0% of dry weight) are smaller than the celery leaf (2.6%) and spinach (2.6%), but higher than on lettuce (1.3%) and Chinese cabbage (1.4%). Regarding, *S. herbacea* it is reported to contain NaCl and other minerals, particularly magnesium, calcium, iron, potassium iodide, as well as dietary fibers (25,29). Additionally, it contains essential amino acids, betaine amino acid (a methanol extract of this species contained 4.85 mg/mL) and choline.

1.1.3.1. Lipophilic Compounds

Lipophilic compounds are insolubility in water (30). These compounds shows several functions such as structural functions in cells, storage and releasing of fuel under stress conditions and hormonal response, and are also involved in numerous biological processes, such as transcription of the genetic code, regulation of vital metabolic pathways and physiological responses (31).

Besides that, some lipids reduce the susceptibility for some diseases, which is the main reason why scientists focus their attention in this group of compounds. Lipids play an important role on many human processes, such as obesity management and consequent weight loss, they can either act in plasma lipid high and low concentrations. The lipophilic composition of *Salicornia* species generally consists in fatty acids, carotenoids, tocopherols and sterols, being described below.

Fatty acids

Fatty acids are carboxylic acids, that present a long aliphatic chain and they might be saturated or unsaturated (32,33). The unsaturated fatty acids differ from saturated fatty acids by presenting double bonds in their chain. Almost all naturally occurring unsaturated fatty acids have the double bonds in the *cis* configuration. The unsaturation provides some physical properties to these fatty acids, such as: the double bonds forces a kink (or more than one) into the hydrocarbon chain. Therefore, these fatty acids cannot pack as tightly as the completely saturated ones, their interactions with each other are weaker and they remain liquid at room temperature (34). The ω-3 (omega-3), ω-6 (omega-6), ω-9 (omega-9) are some of the main groups of fatty acids, for example, the linoleic acid (C18:2) an ω-6, and α-linolenic acid (C18:3), an ω-3, are essential polyunsaturated fatty acids that must be obtained from the diet. These essential fatty acids are required since the human body lacks enzymes (saturases) that have the capacity to biosynthesize them (32).

Saturated fatty acids occur in higher amounts than polyunsaturated acids in *S. ramosissima* (20), being palmitic acid, the most abundant compound. Many oil parameters of *S. bigelovii* seeds are comparable to safflower oil (27) and a study determined a lipid profile similar in *S. europaea* (35). Oil analysis of their seeds indicates that the total content of fat ranges between 26 and 30%, being linoleic acid the main component (approximately 70% of the fatty acid content) (35,36). Figure 7 shows the

unsaturated fatty acids reported on *Salicornia* species and Table 2, the content of fatty acids in *Salicornia* species.

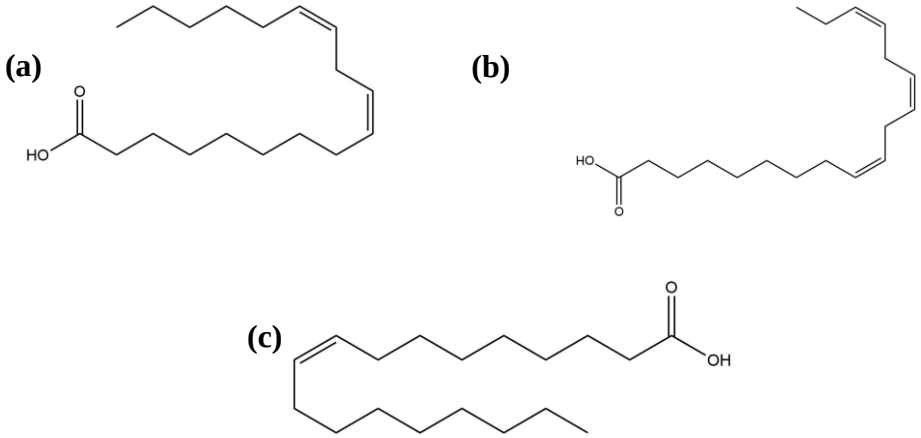

Figure 7 - Unsaturated fatty acids reported on *Salicornia* species; (a) – Linoleic acid; (b) – Linolenic acid; (c) – Oleic acid.

Due to the growing interest in polyunsaturated fatty acids (PUFA's), which provide health benefits, it´s imperative to emphasize that linolenic and linoleic acids are present in *S. ramosissima* (20) and the utilization of this plant might contribute to the consumption of this polyunsaturated fatty acids, being the amounts of these compounds present on Table 2.

Phytosterols

Sterols are characterized by a four-ring core structure called steroid nucleus. Similar sterols are found in plant tissues named as phytosterols, for example stigmasterol (34). These phytosterols are characterized by extra carbon substituents on the side chain attached at C-24 and they have an important role in human health since they inhibit cholesterol absorption (32,33).

The effects of phytosterols in human health are mostly associated with non-pharmacological lipid-lowering therapies, reducing exogenous cholesterol absorption by showing a competition for incorporation into the micelle against cholesterol since they are more hydrophobic. Because of this physical-chemical propriety, both total cholesterol and HDL are reduced. A consumption of 1.8-2.0 g of phytosterols per day has been related to lower cholesterol absorption (37). There are some sterols reported and quantified in *Salicornia* species, namely stigmasterol, 24-ethyl-d(22)-coprostenol, β-sitosterol,

stigmast-7-en-3-ol and stigmastanol. The content of these sterols in *Salicornia* species are shown in Table 2 and the most abundant are shown in Figure 8.

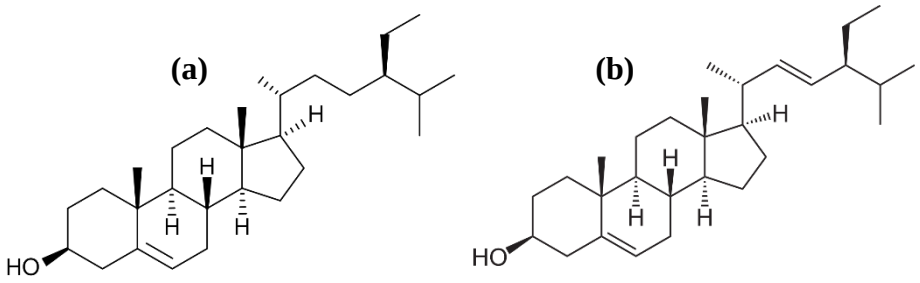

Figure 8 - Most abundant phytosterols; (a) – β-sitosterol; (b) - stigmasterol

Carotenoids and Vitamin A

Carotenoids (C40) are widespread in plants and fruits since they play a role in photosynthesis but they can also be found in fungi and bacteria. In photosynthesis, they act as accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus. These compounds are considered important antioxidant molecules in humans since they diminish cell damage by quenching singlet oxygen and scavenging peroxyl radicals. Some important metabolites of these tetraterpenes are the A group of vitamins, for example, retinol or Vitamin A1, it is derived by oxidative metabolism in mammals mostly from β-carotene (32). The carotenoids content in some *Salicornia* species was already determinate, being represented in Table 2.

Vitamin E

Vitamin E is a fat-soluble vitamin that exists in eight different forms of two different classes: tocopherols and tocotrienols. These compounds are widely disperse in plants and for each class (tocopherols and tocotrienols) of vitamin E, they present four different types (32). These two classes of compounds are structurally similar: they have a chromanol ring with a hydroxyl group and a hydrophobic side chain. Tocopherols includes a chromanol ring and an attached isoprene derived side chain with 16 carbons and they play an important role in the prevention of lipid peroxidation (38,39). The four tocopherols (α, β, γ and δ) differ in the number and position of the methyl groups on the chromanol ring (40). Vitamin E, in humans, it is preferentially accumulated in α-tocopherol form and the functions of this vitamin are several being the prevention of oxidation, the main one (30,39). Besides this, vitamin E play an important role in the

conversion of arachidonic acid to prostaglandins and in the aggregation of blood platelets, slowing down this process (30). The main tocopherols reported in *Salicornia* species are represented in Figure 9.

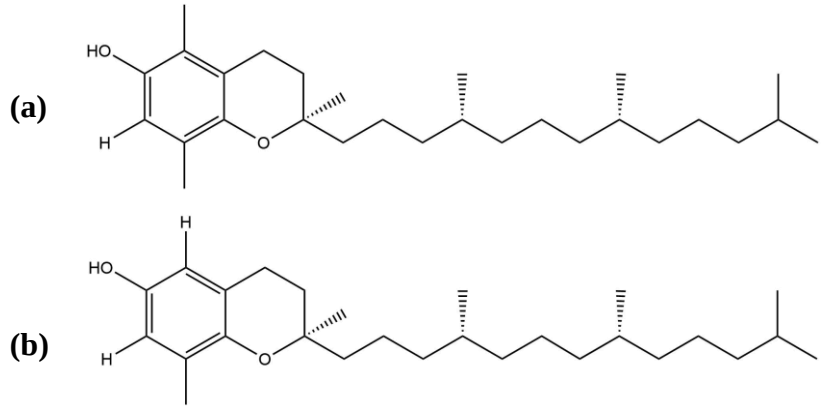

Figure 9 - Tocopherols reported in *Salicornia* species; (a) – α-tocopherol; (b) – δ-tocopherol.

A summarized table with an overview of lipophilic compounds is shown below (Table 2).

Table 2- Lipophilic compounds presented in *Salicornia* species.

	***Salicornia* species content (g/100g)**				
	S. ramosissima extract (plant)	*S. herbacea* Seeds	*S. europaea* seeds	*S. bigelovii* Seeds	*S. persica* plant
Fatty acids					
Saturated					
C9:0	0.21±0.01[1]	_____	_____	_____	_____
C10:0	0.12±0.01[1]	0.03[9]	_____	_____	_____
C12:0	0.20±0.03[1]	0.04[9]	_____	_____	_____
C14:0	0.88±0.01[1]	0.13[9]	_____	0.178[4]	_____
C15:0	0.14±0.01[1]	0.02[9]	_____	_____	_____
C16:0	12.08±0.28[1]	11.84±0.45[9]	6.9 ± 0,9	7.52 ± 0.24[3] 8.504[4]	_____
C17:0	0.24±0.07[1]	0.14±0.02[9]	_____	_____	_____
C18:0	1.00±0.07[1]	3.07±0.47[9]	0.9 ± 0.2	1.45 ± 0.07[3]	_____
C20:0	1.04±0.04[1]	13.52±0.47[9]	_____	6.59[4]	_____
C21:0	0.43±0.07[1]	0.89±0.05[9]	_____	_____	_____
C22:0	2.67±0.13[1]	2.52±0.13[9]	_____	_____	_____
C23:0	1.40±0.06[1]	0.03[9]	_____	_____	_____
C24:0	3.02±0.07[1]	1.53±0.09[9]	_____	_____	_____
C26:0	1.88±0.20[1]	_____	_____	_____	_____

***Salicornia* species content (g/100g)**					
	S. ramosissima extract (plant)	*S. herbacea* Seeds	*S. europaea* seeds	*S. bigelovii* Seeds	*S. persica* plant
Saturated					
C28:0	4.37±0.22[1]	_____	_____	_____	_____
C30:0	1.57±0.12[1]	_____	_____	_____	_____
Unsaturated					
C16:1 (9E)	0.20±0.03[1]	0.01[9]	_____	_____	_____
C16:1 (9Z)	0.40±0.04[1]	_____	_____	_____	_____
C18:2 (9Z, 12Z)	1.25±0.19[1]	43.73±1.23[9]	71.1 + 1.3[2]	75.50 ± 2.04[3] 63.4[4]	_____
C18:3 (9Z, 12Z, 15Z)	0.53±0.11[1]	0.35±0.02[9]	_____	1.98 ± 0.09[3] 1.34[4]	_____
C18:1 (11E)	0.69±0.12[1]	_____	_____	_____	_____
C18:1 (9Z)	0.22±0.01[1]	19.81±0.86[9]	21.8 + 0.8[2]	13.42 ± 0.56[3] 19.985[4]	_____
Total UFA	3.29[1]	63.9[9]	92.9[2]	90.9[3] 84.725[4]	_____
Total SFA	31.3[1]	32.8[9]	7.8[2]	8.97[3] 15.272[4]	_____
Sterols		seeds/plant			
β-sitosterol	3.17 ± 0.07[1]	✓[10] (plant) 0.00945±0.0005[9]	✓[11] (plant)	✓[11] (plant)	_____
Stigmastanol	2.21 ± 0.07[1]	_____	✓[11] (plant)	✓[11] (plant)	_____
Stigmasterol	3.71 ± 0.10[1]	✓[8] (plant) 0.00657±0.0003[9]	✓[11] (plant)	✓[11] (plant)	_____
24-ethyl-d(22)-coprostenol	1.07 ± 0.25[1]	_____	_____	_____	_____
Stigmast-7-en-3-ol	1.64 ± 0.07[1]	_____	_____	_____	_____
Ergosterol		✓[8] (plant)	_____	_____	
Sitostanol			✓[11] (plant)	✓[11] (plant)	
Tocopherols					
α-tocopherol	0.00114 ± 0.0007[7]	0.02492±0.0003[9]	_____	0.02 ± 0.0013[3]	_____
γ-tocopherol	_____	0.00756±0.0003[9]	_____	0.0075 ± 0.0005[3]	_____
δ-tocopherol	0.00077 ± 0.00005[7]	0.00893±0.0003[9]	_____	0.0063 ± 0.0005[3]	_____
β-carotene	_____	0.00945±0.0004[9]	_____	0.016 ±0.006[6]	0.0047[5]

[1]Reference (20); [2]Reference (35); [3] Reference (27); [4]Reference (41); [5]Reference (42); [6]Reference (28); [7] Reference (5); [8]Reference (43); [9]Reference (44); [10]Reference (45); [11]Reference (46) ✓- only identified;

1.1.3.2. Phenolic Compounds

Phenolic compounds are omnipresent in plants, showing a diversity of structures and represent an important role in growth, reproduction and development of the plant, they are also responsible for color and sensory characteristics, such as flavor properties (47). These compounds are also related to health benefits associated to ingestion of fruits and vegetables (both have these compounds in their composition). Some studies reveal that phenolic compounds potentially show protective effects, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects. These health benefits are also associated to their antioxidant activity which is determined by the chemical structure (47–49). The structural arrangements with greatest antioxidant activity in flavonoids are the ortho 3',4'-dihydroxy moiety in the B ring, the meta 5,7-dihydroxy arrangements in the A ring and the 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization, as long as the o-dihydroxy structure in the B ring is also present (Figure 10) (50).

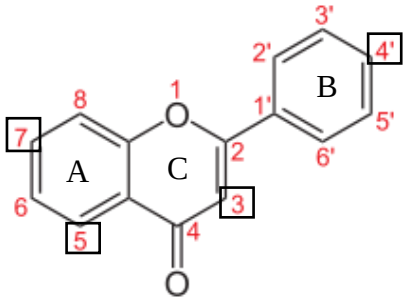

Figure 10 - Structure of a flavonoid.

The information about the phytochemical content is imperative to develop further applications for this halophyte plant. Phenolic fraction composition is already described for *S. herbacea* species. However, the quantification of phenolic compounds is not reported. The structures of the most common detected phenolic compounds (phenolic acids and derivatives as well as flavonoid) in *Salicornia* species are represented in Figure 11 and an overview of all phenolic compounds and total phenolic and flavonoid content reported for Salicornia species is represented in Table 3.

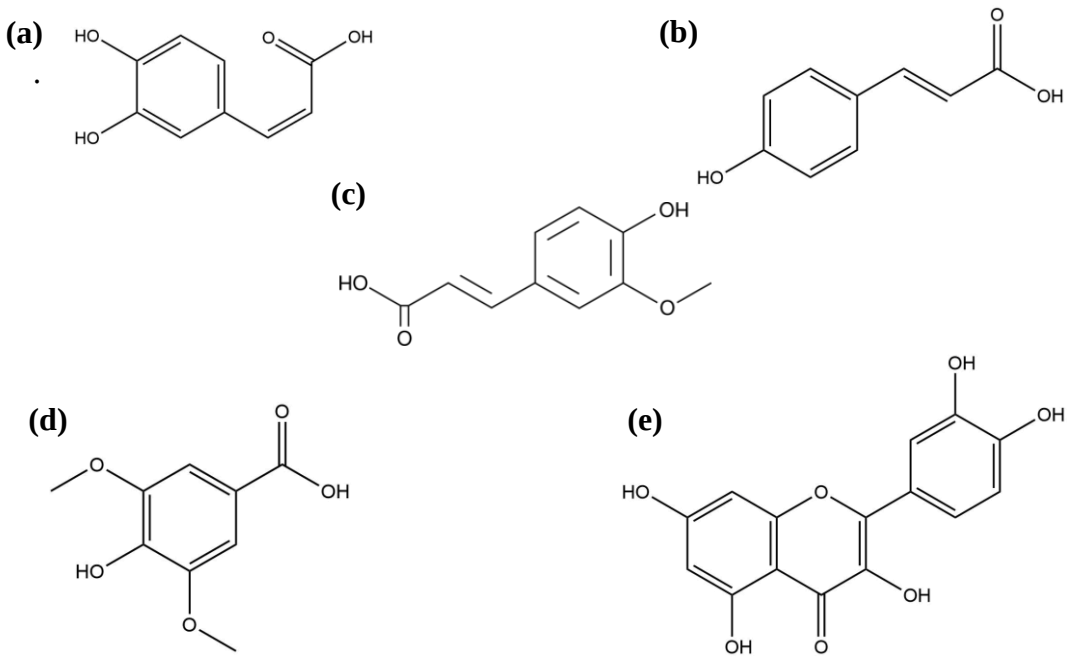

Figure 11 - Structures of the most common phenolic compounds detected in *Salicornia* species; (a) – caffeic acid; (b) – p-coumaric acid; (c) – ferulic acid; (d) – syringic acid; (e) – isorhamnetin.

Table 3 – Phenolic compounds and total phenolic and flavonoid content reported for *Salicornia* species.

	Species							
Phenolic acids and derivatives	*S. ramosissima*	*S. europaea*	*S. herbacea*	*S. freitagii*	*S. brachiata*	*S. perennans*	*S. crassa*	*S. patula*
Caffeic acid	✓[1]	✓[4]	✓[5]	✓[6]	-	-	-	-
Caffeoylquinic acid	✓[1]	✓[4]	✓[5]	✓[6]	-	-	-	-
Caffeoyl-hydrocaffeoylquinic acid	✓[1]	-	-	-	-	-	-	-
Dihydrocaffeoyl quinic acid	✓[1]	-	-	-	-	-	-	-
Dicaffeoyl quinic acid	✓[1]	-	-	-	-	-	-	-
Epigallocatechin gallate	-	-	-	✓[6]	-	-	-	-
Ferulic acid	-	✓[4]	✓[5]	-	-	-	-	-
Gallic acid	-	-	-	✓[6]	-	-	-	-
Hydrocaffeic acid	✓[1]	-	-	-	-	-	-	-
Hydrocaffeoylquinic acid	✓[1]	-	-	-	-	-	-	-
Procatechuic acid	-	-	✓[5]	-	-	-	-	-
p-Coumaric acid	-	✓[4]	✓[5]	✓[6]	-	-	-	-
Rosmarinic acid	-	-	-	✓[6]	-	-	-	-
Syringic acid	-	-	✓[5]	✓[6]	-	-	-	-
Salicylic acid	-	-	✓[5]	-	-	-	-	-
Sinapic acid	-	-	✓[5]	-	-	-	-	-
Scopoletin	✓[2]	-	✓[8]	-	-	-	-	-
Trans-cinnamic acid	-	-	✓[5]	-	-	-	-	-
Vanillic acid	-	-	-	✓[6]	-	-	-	-
Flavonoids								
Acacetin	-	-	✓[5]	-	-	-	-	-

	Species							
Flavonoids	*S. ramosissima*	*S. europaea*	*S. herbacea*	*S. freitagii*	*S. brachiata*	*S. perennans*	*S. crassa*	*S. patula*
Epicatechin	-	-	-	✓[6]	-	-	-	-
Galangin	-	-	✓[5]	-	-	-	-	-
Hesperetin	-	-	✓[5]	-	-	-	-	-
Isorhamnetin	✓[1]	-	✓[5]	-	-	-	-	-
Isorhamnetin glucoside	✓[1]	-	✓[9]	-	-	-	-	-
Kampferol	-	-	✓[5]	-	-	-	-	-
Myricetin	-	-	✓[5]	-	-	-	-	-
Quercetin glucoside	✓[1]	-	✓[9]	-	-	-	-	-
Quercetin	-	-	✓[5]	-	-	-	-	-
Rutin trihydrate	-	-	-	✓[6]	-	-	-	-
Rhamnetin	-	-	✓[5]	-	-	-	-	-
Total phenolic content	2744 ± 68[1]	-	-	913 ±51[6]	890[7]	918 ± 61[6]	891 ± 107[6]	1037 ± 44[6]
(mg GAE/100g dw)	3300 ± 70[3]			918± 98[6]		994 ± 57[6]		
Total flavonoid content	969 ± 28[1]	-	-	129 ± 3[6]	740[7]	82 ± 8[6]	139 ± 9[6]	112 ± 1[6]
(mg CE/100g dw)	1750 ± 210[3]			116 ± 2[6]		47 ± 2[6]		

[1]Reference (51); [2]Reference (52); [3]Reference (5); [4]Reference (53); [5]Reference (26); [6]Reference (54); [7]Reference (55); [8]Reference (43); [9]Reference (71);
dw – dry weight;
GAE – gallic acid equivalents;
CE – quercetin equivalents;

Beyond the presented phenolic compounds in Table 3, there were also reported in *S. herbacea* four dicaffeoylquinic acid derivatives, 3-caffeoyl-5-dihydrocaffeoylquinic acid, 3-caffeoyl-5-dihydrocaffeoylquinic acid methyl ester, 3-caffeoyl-4-dihydrocaffeoylquinic acid methyl ester and 3,5-di-dihydrocaffeoylquinic acid methyl ester (56). In addition to this, some chromones and flavanones derivatives were also found in this specific species (57).

1.1.3.3. Minerals

Minerals are essential nutrients that contribute to maintain body health (58,59) and the essential minerals reported for *Salicornia* species are represented in Table 4.

Table 4 – Essential minerals in different *Salicornia* species.

	Minerals							
Mineral	***S. ramosissima*** (mg/g) DW (5)	***S. herbacea*** (mg/g) FW (25)	***S. freitagii*** (mg/g) DW (54)	***S. bigelovii*** (mg/g) FW (28)	***S. perennans*** (mg/g) DW (54)	***S. crassa*** (mg/g) DW (54)	***S. patula*** (mg/g) DW (54)	**Recommended daily intake levels**
Na	89.9 ±0.5	12.181	(1) 145.0 ± 0.3 (2) 193.0 ± 0.2	9.98 ± 0.71	(1) 179.0 ± 0.6 (2) 197.0 ± 0.5	202 ± 0.3	141.0 ± 0.4	1500 mg
Ca	4.86 ± 0.05	1.588	____	0.62 ± 0.02	____	____	____	1000 mg
K	8.92 ± 0.23	7.401	(1) 24.7 ± 0.4 (2) 16.7 ± 0.2	1.76 ± 0.08	(1) 27.9 ± 0.8 (2) 18.9 ± 0.1	19.9 ± 0.6	25 ± 0.4	4700 mg
Mg	9.43 ± 0.08	0.540	(1) 8.5 ± 0.3 (2) 2.7 ± 0.1	1.18 ± 0.06	(1) 5.2 ± 0.1 (2) 3.1 ± 0.1	0.68 ± 0.02	6.6 ± 0.1	450 mg
Zn	0.0687 ± 0.0001	0.296	____	0.00405± 0.00014	____	____	____	8-11 mg
Fe	1.53 ± 0.02	0.662	____	0.01 ± 0.00	0.031 ± 0.002	____	0.014 ± 0.001	8 mg
Cu	____	0.011	____	0.00091 ± 0.00014	____	____	____	900 µg
Ni	0.00191 ± 0.0001	0.007	____	____	____	____	____	< 100 µg
Mn	0.204 ± 0.004	0.039	____	____	____	____	____	1.8-2.3 mg

The recommended daily intake levels are for adults according to (60).
– not documented

The most abundant minerals documented in *Salicornia* species are Na, K, Mg and Fe. Besides that, in a recent work, *S. ramosissima* showed the possibility to be a source of Mn, 60 g of fresh *S. ramosissima* would correspond to an intake of 2 mg of Mn recommended by the WHO without exceeding the maximum amount of sodium recommended by the same organization (5). This mineral exhibits some important benefits, including healthy bone structure, bone metabolism and it helps to create essential enzymes for building bones. Besides, there are other health benefits of this mineral including the formation of connective tissues, absorption of calcium, proper functioning of the thyroid gland and sex hormones, regulation of blood sugar level and metabolism of fats and carbohydrates (61). Some evidences indicate that recommended dietary intakes of certain vitamins and minerals such as calcium, folic acid and selenium reduce the risk of certain diseases (62). Deficiencies of these recommended dietary intakes for essential nutrients can contribute to appearance of some diseases (63,64), nutrition is important for bone health, being some minerals and vitamins associated to the prevention of bone diseases, particularly osteoporosis (65).

Nowadays, the higher consumption of sodium has gained more attention, since this mineral is associated to high blood pressure that can result in heart diseases (66). The fact that this mineral is present in lower amounts in salt, might help in the reduction of this mineral intake. Furthermore, when located in polluted zones, halophytes can accumulate metals like Zn, Cr, Pb, Ni and Cd (67). The highest concentrations of these metals are usually found in the roots, as determined in some halophytes species (67,68). The Table 4 resumes of the mineral composition of some *Salicornia* species.

However, there are some factors that can affect the chemical composition of *Salicornia* species. The morphology and genetic composition of different *Salicornia* species (3), the different physiological stages that might change not only the components but also the levels of the chemical compounds present. The vegetative stage of this glasswort is the most documented in the literature, being the fructification stage the less studied, almost not mentioned (6,17). There are some studies in seeds of different species of *Salicornia* as well, where it is visible the differences at fatty acids composition when compared to the vegetative stage (20,35).

The location can also affect the chemical composition of the plants, since every plant shows an optimal growth conditions and when these conditions are altered, the plant

can be exposed to different stress and produce different compounds or in different quantities (54,69). For halophyte plants, one of the most stress associated it is the salt levels present in the soil. There are several studies about the optimal conditions associated to this halophyte, essentially associated at different salt concentrations of the soil (6,70,71). These facts induces that the environment associated to the germination and growth of the plant might influence the chemical composition and the bioactive compounds present as some studies reported (69). Differences in chemical composition of this species are mentioned bellow. The same species, *S. perennans,* collected in different locations at the same time periods showed some differences. For example, the total flavonoid content of one of the samples was almost the double of the other (54). This fact induces that the same species can have different chemical composition and different amounts of some bioactive compounds based on the location and the environment associated. Furthermore, it was reported that the total phenolic content under heat and salt stresses decreased when compared to control conditions. One the other hand in the same study, the total flavonoid content increased in both stresses conditions when compared with the control conditions, being the heat stress, the one with more total flavonoid content (69).

1.1.4. Evaluation of biological activities on *Salicornia* species

1.1.4.1. Potential health benefits

Salicornia herbacea has been used as folk medicine in a variety of diseases such as obesity, diabetes and asthma. There are few studies on their pharmacological and biological effects that reports the anti-inflammatory, antiproliferative (72), antihyperglycemic (73), antioxidant (29,74,75) and antihyperlipidemic activity of this species (76). An overview of biological activities reported is shown below, in Table 5.

Table 5- An overview of biological activities reported on extracts and isolated compounds from *Salicornia* species extracts.

Species	Extract/ compound	Concentration	Biological activity	Results	Ref
S. herbacea	(isorhamnetin 3-*O*- glucoside isolated)	0.1; 5.0; 10.0 μg/mL	*in vitro* effect on NO production and protein expression of pro-inflammatory mediators in LPS-stimulated Raw 264.7	Isorhamnetin 3-*O*-glucoside suppressed inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and IL-1β (interleukin 1 beta).	(77)
	(isorhamnetin 3-*O*- glucoside isolated)	0.5; 1.0; 5.0; 10.0 μM	*in vitro* protective effect against oxidation-induced cell damage.	Isorhamnetin 3-*O*-glucoside elevated GSH level, inhibited oxidative damage of purified genomic DNA and suppressed activity of myeloperoxidase (MPO) in tumor necrosis factor-α (TNF-α) stimulated human myeloid cells.	(78)
	Extract (plant) (50% ethanol)	*Salicornia* (SH): 350 mg/Kg and 700 mg/Kg Metformin 250 mg/Kg	*in vivo* effects of *Salicornia* extracts in ICR mice with high fat diet-induced hyperglycemia and hyperlipidemia.	The insulin resistance indices of SH350 and SH700 groups were reduced by 27% and 25%, respectively. SH350 and SH700 showed a reduction of the NEFA, TG, TC, and LDL-C levels. Metformin showed similar results when compared to SH700 group.	(79)
	(isorhamnetin 3-*O*-glucoside, and quercetin 3-*O*-glucoside isolated)	0.1; 1.0; 5.0; 10.0 μM	*in vitro* effects of flavonoid glycosides on matrix metalloproteinase (MMP-9 and MMP-2) in HT1080 cells	Isorhamnetin 3-*O*-glucoside in these HT1080 cells inhibited significantly MMP-9 and MMP-2 activities. 3-*O*-glucoside decreased significantly the expression level and activity of MMP-9. MMP-9 and MMP-2 activities exhibited dose-dependent pattern (10 μM).	(72)

Species	Extract/ compound	Concentration	Biological activity	Results	Ref
S. herbacea	Extract (plant) (aqueous)	100 mg/Kg	*in vivo* effect in Ovariectomy-Induced Oxidative Stress.	Free radical-scavenging activity of SH was measured (IC_{50} DPPH= 197.29 µg/mL IC_{50} $\cdot O_2^-$= 21.06 µg/mL); The MDA content was inhibited by 94.12%. The extract increased the activation of SOD in the liver total homogenate and mitochondrial fraction and restored the decrease in CAT levels. The antioxidative effect of the extract was supported by the histopathologic examination	(80)
	(isorhamnetin 3-*O*-glucoside isolated)	0.1; 5.0; 10.0 µM 25 or 50 mg/Kg	*in vitro* effects on Rat Lens Aldose Reductase and *in vivo* effects on sorbitol Accumulation in streptozotocin-induced diabetic Rat Tissues.	IC_{50} =1.4 µM (inhibitory effect on aldose reductase); The inhibitory effect in sorbitol accumulation was 44.2% and 21.8% in red blood cells and sciatic nerves, respectively.	(73)
	(isorhamnetin 3-*O*- glucoside and quercetin 3-*O*-glucoside isolated)	10; 20 µM	*in vitro* effects on anti-adipogenic activity.	The flavonoids showed significant suppressive effect on adipogenic differentiation. Especially, quercetin 3-*O*-glucoside.	(81)
	Extract (plant) (ethanol)	1% (wt/wt)	*in vivo* hypolipidemic effect on animal model of type 2 diabetes mellitus.	The extract reduced plasma triglyceride and cholesterol in animal models of type 2 diabetes.	(82)

Species	Extract/ compound	Concentration	Biological activity	Results	Ref
S. herbacea	Vinegar with *S. herbacea* (24g of powder)	7g/Kg	*in vivo* anti-fatigue effect in rats exhausted by exercise.	The vinegar showed anti-fatigue effect by promoting lactate metabolism and maintaining ATP levels in rats exhausted by exercise.	(83)
S. freitagii	Extract (plant)	-	*in vitro* antioxidant activity	IC_{50} DPPH = 2.54±0.22 IC_{50} DPPH = 3.89±0.15	(54)
S. perennans	Extract (plant)	-	*in vitro* antioxidant activity	IC_{50} DPPH = 3.75±0.05 IC_{50} DPPH = 5.39±0.13	(54)
S. crassa	Extract (plant)	-	*in vitro* antioxidant activity	IC_{50} DPPH = 2.91±0.14	(54)
S. patula	Extract (plant)	-	*in vitro* antioxidant activity	IC_{50} DPPH = 3.88±0.33	(54)
S. ramosissima	Extract (plant) (ethanol)	_____	*in vitro* antioxidant activity	IC_{50} DPPH = 5.69 ± 0.09 mg/mL IC_{50} NO >10 mg/mL IC_{50} FRAP >10 mg/mL	(5)
	Extract (plant) (ethanol)	50 mg/Kg	*in vivo* effects on mouse testis under toxicological conditions.	The results showed that, statistically, no significant effect ($p>0.05$) were observed on testicular weight, volume and gonadosomatic index among groups treated; The protective action of the extract could only be evidenced at the histopathological level.	(52)

LPS - lipopolysaccharide; TC – total triglyceride; TC - total cholesterol; NEFA - non-esterified fatty acids; GSH – glutathione; LDL-C - low-density lipoprotein; ICR - institute for cancer research; HT1080 - fibrosarcoma cell line; ATP - adenosine triphosphate; SOD - superoxide dismutase; CAT – catalase;

In addition to these facts, nowadays, there is a worldwide concern with excessive consumption of salt (9-12g/day), being the reduce of salt and sodium intake levels, one of the global targets until 2025 (reduce 30%) (84,85). The excessive consumption of salt has been related to some diseases, such as hypertension, cardiovascular disease and renal dysfunction, so, because of that, it is imperative to develop new solutions (8,86,87). *S. herbacea* showed potential to be used as salt substitute (88), prove of that is the patents applications as "Preparation of nutrient rich salt of plant origin" and "*Salicornia* spp.-Derived Salt and Its Production Process" (4,89). Many studies have focused on the health benefits of *Salicornia herbacea* (Table 5). However, *S. ramosissima* among other species are not well explored. Thus, in order to explore the potential health benefits for this species, *in vitro* antioxidant and anti-inflammatory assays must be performed.

1.1.4.2. *in vitro* antioxidant and anti-inflammatory assays

An imbalance of the pro-oxidant-antioxidant equilibrium in favor of the pro-oxidants results in an oxidative stress. Under normal conditions, the effects of oxidants are prevented by the antioxidant system, consisting in enzymatic and nonenzymatic antioxidants (90). Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), among others, are examples of human antioxidant system. However, when they are insufficient, exogenous antioxidants are required (91). Oxidative stress may result in an increased lipid peroxidation, deoxyribonucleic acid (DNA) damage, protein damage, among others (92). These processes can induce a variety of diseases (e.g. cancer, neurological, arteriosclerosis, diabetes) as well as ageing and inflammation processes (93,94). Therefore, measurement of antioxidant activity in plants allows to infer their potential inhibition or scavenging capacity against ROS. Thus, to evaluate the radical scavenging activity of *S. ramosissima* extracts, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), 2,2-azobis (3-ethyl-benzothialzoline-6-sulfonic acid) ($ABTS^{+\cdot}$) and nitric oxide assays were performed. Ability to prevent lipid peroxidation and ferric reducing antioxidant power (FRAP) assays were also evaluated.

The $DPPH^{\cdot}$ assay allows to quantify the radical scavenging activity by testing if the extracts can react with the radical 2,2-Diphenyil-l-picrylhydrazyl ($DPPH^{\cdot}$). The reduction of the radical, represented in Figure 12 can be measure by the decreasing of the absorbance (95).

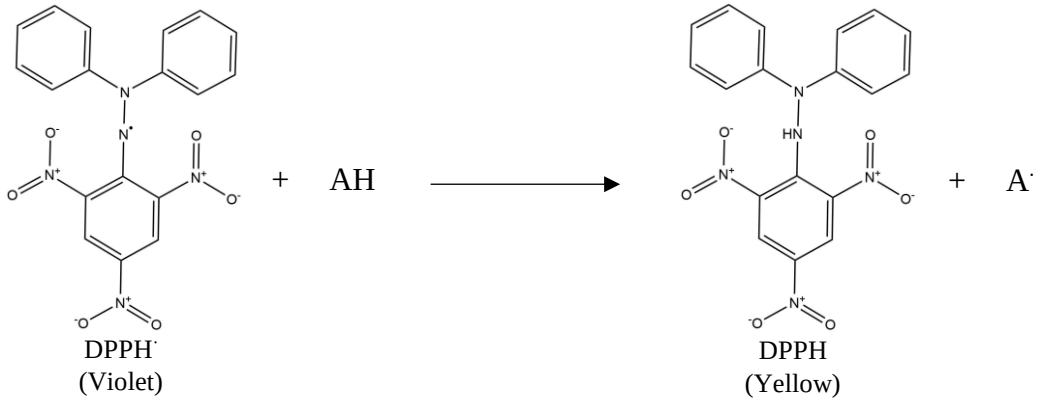

Figure 12 – DPPH· radical scavenging by an antioxidant (AH); Adapted from (96).

In the ABTS assay, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid**)** (ABTS) is converted to its radical cation ($ABTS^{+\cdot}$) and it can react with antioxidants, forming again ABTS (colorless product) (97).

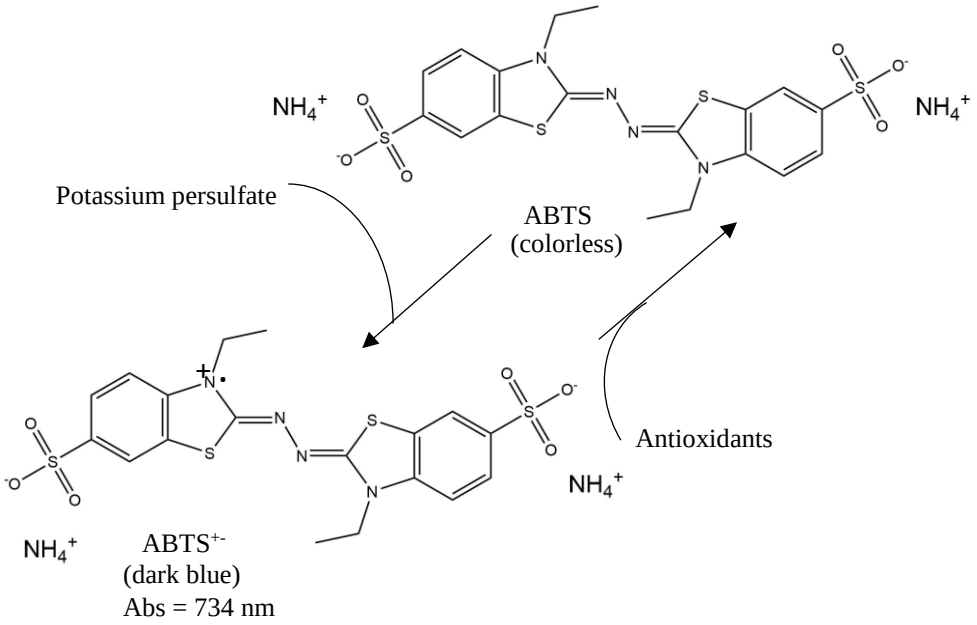

Figure 13- ABTS chemical reaction; Adapted from (98).

Furthermore, nitric oxide (NO) is an important molecule involved in many physiological and pathological processes. NO is produced from amino acid L-arginine by vascular endothelial cells and macrophages. The reaction of this radical with superoxide radical, forms a highly reactive peroxynitrite anion ($ONOO^{-}$), that can cause oxidative damage (99). The antioxidants from natural sources can be the alternative to synthetic

antioxidants, thus the NO scavenging assay was performed in *S. ramosissima* extracts. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize with sulfanilamide and couple with N-(1-naphthyl)ethylenediamine dihydrochloride, forming pink colour, which was measured at 546 nm. The antioxidants donate protons to the nitrite radical and the absorbance decreased. This decrease measures the extent of nitrite radical scavenging (100).

Lipid peroxidation (LP) is a process of oxidative degradation of lipids, manly, polyunsaturated fatty acids by reactive oxygen species, leading to damage in cells. This process consists in three important steps: initiation, propagation and termination. The steps are represented in Figure 14, the initiation reaction occurs when the radical (ex. ·OH) attacks the most reactive hydrogen (near the double bound), leading to the formation of a fatty acid radical (101).

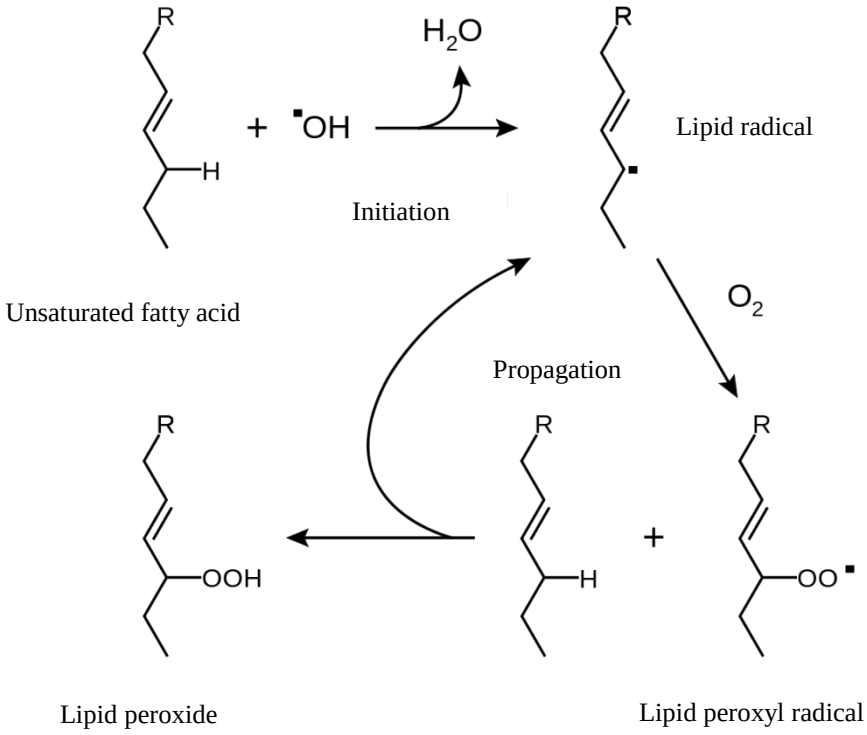

Figure 14 – Reactions of lipid peroxidation; Adapted from (102).

The radical formed is unstable and can react with a triplet oxygen, producing a fatty acid peroxyl radical. This radical can react with another polyunsaturated fatty acid, leading to the formation of new fatty acids radicals. The propagation step ends when peroxyl radicals are transformed into non-radical compounds (termination step). One of

the most common final product of lipid peroxidation is malondialdehyde (MDA) (101). The thiobarbituric acid (TBA) assay is based on the reaction of thiobarbituric acid with MDA, under conditions of high temperature and acidity to generate a colored adduct that is measured at 532 nm (Figure 15) (103).

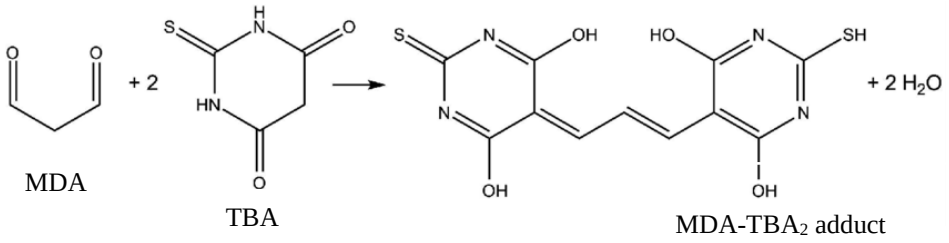

Figure 15 - Reaction of malondialdehyde (MDA) with 2 molecules of 2-thiobarbituric.

Ferric reducing antioxidant power (FRAP) assay takes advantage of electron-transfer reactions. This method measures the reduction of [Fe(III)(2,4,6-tripyridyl-*s*-triazine)$_2$]$^{3+}$ into [Fe(II)(2,4,6-tripyridyl-*s*-triazine)$_2$]$^{2+}$, a blue colored product (104).

The inflammation process constitutes a response of the organism to tissue damage, being involved in different human diseases, including asthma, diabetes, cardiovascular diseases, metabolic syndrome, hypertension, among others (105). In order to investigate the anti-inflammatory activity, the eicosanoids production from arachidonic acid metabolism can be studied. Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of membranes cells and it is released into the cell by the action of phospholipase A$_2$ (PLA$_2$). This fatty acid is used as substrate to eicosanoids production, that are hormones which are involved in diverse biological effects that act only on cells near the point of hormone synthesis (34). Inflammation, fever and regulation of blood pressure are examples of some of the processes that these hormones are involved (34). There are three distinct pathways to convert arachidonic acid in eicosanoids, cyclooxygenase (leading to the formation of prostaglandins and thromboxanes), lipoxygenase (leading to the formation of leukotrienes) and epoxygenase pathway (leading to cytochrome P-450 and epoxides production) (106). Cyclooxygenase (COX) pathway, occurs in two forms, called COX-1 and COX-2. COX-1 is also known as constitutive enzyme and it is expressed, for example, in blood monocytes and platelets, being involved in normal cellular homeostasis. In the other hand, COX-2 is also known as inducible enzyme and it is expressed is specific conditions, such inflammation

(induced by a pro-inflammatory stimuli) (106). COX-1 can be found in human platelets, initiating the arachidonic acid metabolism, which leads to the production of prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2). The inhibition of this enzyme results in a relief of the inflammation, since, prostaglandins synthesis is significantly increased in inflamed tissues (106). The Figure 16 represent a scheme of the production of PGE_2 and TXA_2 from arachidonic acid.

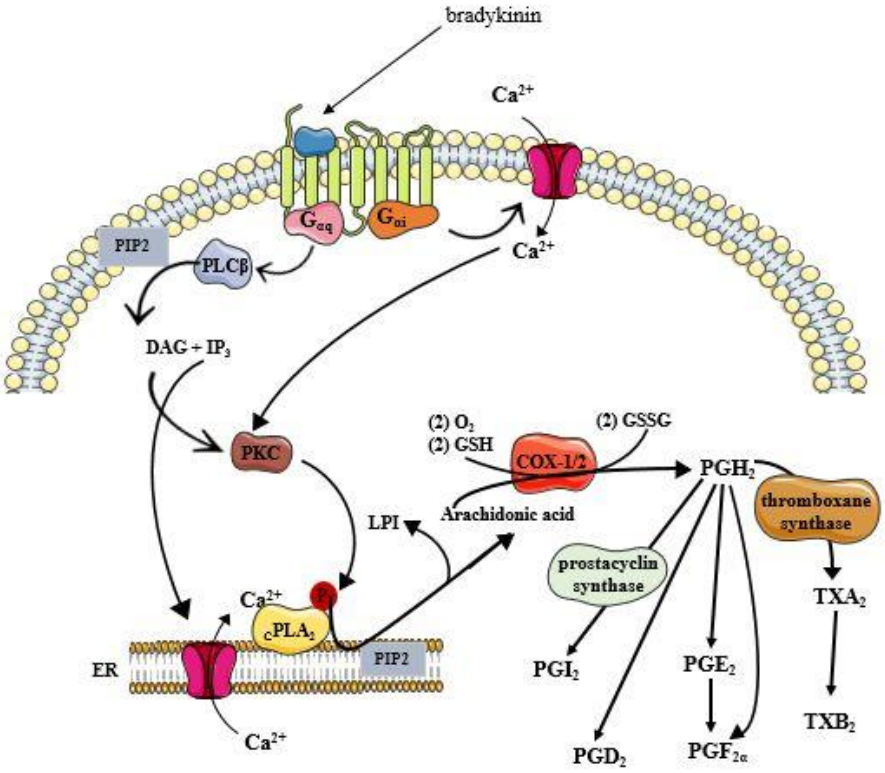

Figure 16 – Prostaglandin (PG) and thromboxane (TX) synthesis from arachidonic acid metabolism. $G_{\alpha q}/G_{\alpha i}$ - G protein subunits; PLCβ - 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta; PIP_2- phosphatidylinositol 4,5-bisphosphate; DAG – diacylglycerol; IP_3- inositol 1,4,5-trisphosphate; PKC- protein kinase C; ER- endoplasmic reticulum; $_cPLA_2$- cytosolic phospholipase A_2; LPI- lysophosphatidylinositol; GSH- reduce glutathione; GSSG- oxidized glutathione; Adapted from (107).

Nowadays, Salicornia species are used as gourmet plant in salads, and more particularly in England, where it is commonly known as "samphire" or "saltwort", it is also consumed as cooked vegetable (108). In order to evaluate their applications, a research was performed and shown in the previous section.

1.2. *Salicornia* species based products in the market

Salicornia spp. have been used as pickles and areal parts of this halophyte plant are added to vinegar (74). Some applications have been related to the fact that it can be a salt substitute, however, the major products from this plant in the market are powder, pills, vinegar, gin, tea and cosmetics (108). Some *Salicornia* spp. based products are demonstrated bellow in Table 6.

Table 6 – *Salicornia* species base products in the market.

	***Gin*** Gin with *Salicornia*. **Origin**: Spain
	***Salicornia* Pill & Powder** Product Origin: Korea Company: DASARANG. Co., Ltd. Product name: Hamcho Plus & Hamcho Fresh
	***Salicornia* Seed** Self-breed variety, break through the natural environment restrictions that *Salicornia* is merely appropriate for growing in the south of north latitude at home and abroad. High purity, germination success rate and oil content percentage. **Origin:** China
	***Salicornia* Tea** Refined of 100% *Salicornia* tender stem. Full of natural plant-salt, alkaloid and some kind of amino acid which human body can't compound naturally. Natural saline taste, easy to be absorbed. **Origin:** China
	**Boiled *Salicornia*** **Origin:** China
	**Cueillette en Baie de Somme** *Salicornia* from "Baie de Somme" with vinegar. **Origin:** France
	***Salicornia* Juice** *Salicornia* juice is a brand-new organic vegetable juice.100% *Salicornia* juice, Delicious and healthy. It can release hypertension, reduce lipemia, lose weight, and improve the metabolism of liver.

- *Salicornia* soup;
- *Salicornia* with vinegar;
- *Salicornia* in their own juice

Origin: France

Soap
It contains *Salicornia herbacea* extract.
Origin: Korea

Soon-ja
Pure cookies with no additives, color or preservative. It contains functional colored rice, shiitake and organic *Salicornia herbacea* for overall nutrition.
Origin: Korea

Moutarde a la SALICORNE
Origin: France, Guérande

Rillettes de Thon
Tuna with *Salicornia*.
Origin: France

Pickles *Salicornia*
Origin: France
Also produced in China and Korea.

Salicornia
Green salt with *Salicornia*.
Origin: **Casa do Vale**
Portugal

Salicornia e Shiitake
Salt with *Salicornia* and Shiitake.
Origin: **Casa do Vale**
Portugal

	**Gin** Gin with *Salicornia ramosissima* **Origin:** Aveiro region Portugal
	**Vinegar** Vinegar with *Salicornia*. **Origin:** Casa do sal/Figueira da Foz Portugal
	**Butter** Butter with *Salicornia*. **Origin:** Casa do sal/Figueira da Foz Portugal
	**Powder** *Salicornia* powder. Origin: Ilha dos Puxadoiros/Ria de Aveiro Portugal
	***Salicornia* spp. plant** **Origin:** Portugal

Table 6 shows that, there are a few products on the market. In this regard, *S. ramosissima* might be a potential matrix in new food formulations. Nowadays, the current market trends are natural and organic food products allied with potential health benefits. Thus, the potential biological active compounds from *S. ramosissima* must be evaluate, in order to develop new food products with potential health benefits.

Chapter II – Screening of lipophilic and phenolic compounds and mineral content present in *Salicornia ramosissima*

Objectives

Considering the interest of phenolic and lipophilic compounds due to their potential health benefits, the present thesis reveals the lipophilic and phenolic profile of *S. ramosissima* from Ria de Aveiro lagoon in a different physiological stage (fructification), since the chemical composition of this stage is not reported.

Furthermore, considering the commercialization and exploitation of *S. ramosissima*, the essential minerals and toxic compounds were also evaluated. The composition of this halophyte was also studied in different locations in Ria de Aveiro lagoon due to the environment effects and the presence of different stress that can condition the chemical composition of the plant.

1. Materials and Methods

The experimental design of the work developed during this chapter is presented in Figure 17.

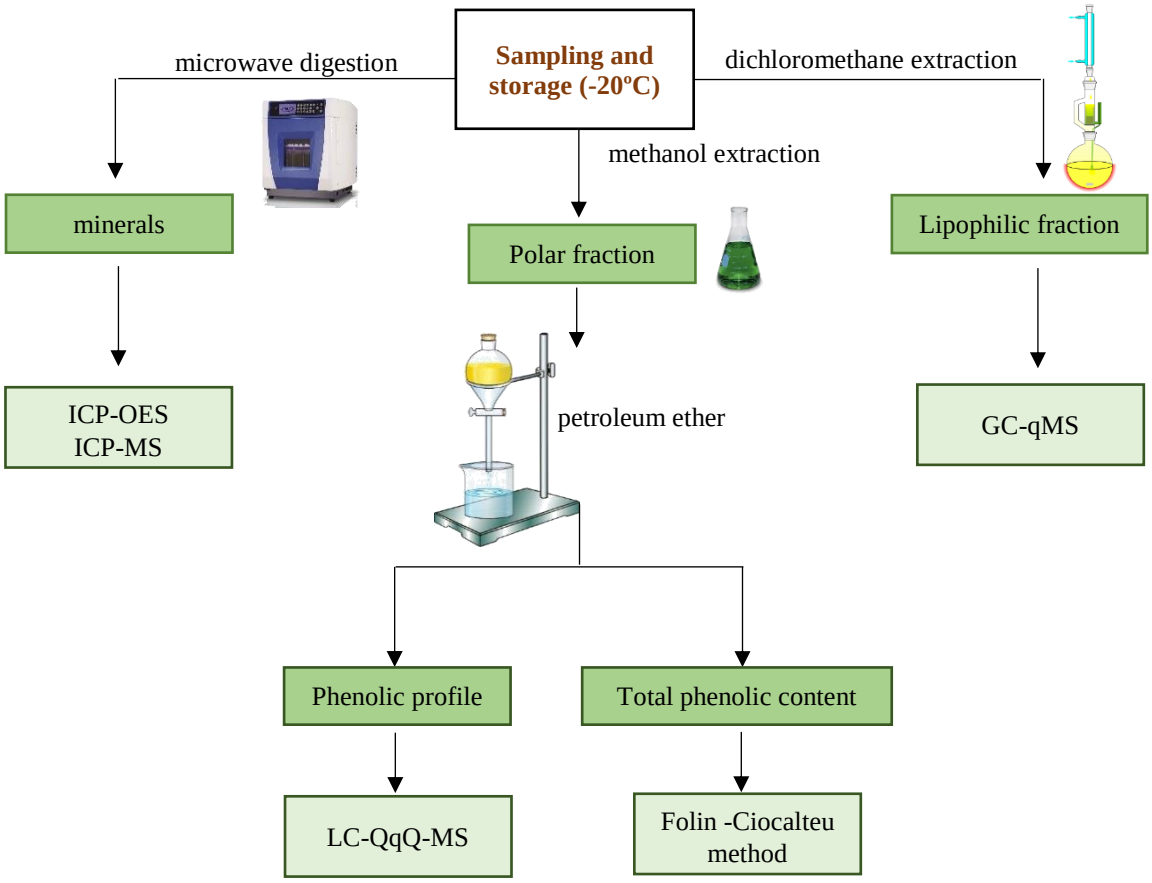

Figure 17 - Experimental design of the work developed in this chapter.

To study the influence of the location and the colour of the plant (red or green) on the total phenolic compounds, phenolic and lipophilic profile, *S. ramosissima* plants were collected in 3 locations. One sample from Marinha dos Peixinhos location was red.

1.1. Sampling

S. ramosissima used in all experiments was collected in Ria de Aveiro lagoon, in Aveiro region, Portugal. The samples identification was performed by Dr. Helena Silva. To study the lipophilic and phenolic composition, the plants were harvested from 3 locations in October and stored frozen (-20ºC) until analysis. Four samples were obtained and the characteristics of the samples locations are described below (Table 7 and Figure 18). During this chapter, the plants of different locations will be identified as BC (Boco river), MP (Marinha dos Peixinhos green), SF (Santiago da Fonte) and MPR (Marinha dos Peixinhos red), accordingly to the harvesting location.

Table 7 – Characteristics of sample locations

	Harvest locations		
	Boco river	Santiago da Fonte	Marinha dos Peixinhos
Longitude	40.535769	40.628086	40.642765
Latitude	-8.664447	-8.662346	-8.659244

Figure 18 – Sample harvest locations; A- Marinha dos Peixinhos; B- Santiago da Fonte; C- Boco river.

S. ramosissima samples used in the experiments were washed several times, dryed at the room temperature and finally stored frozen (-20ºC) until analysis (Figure 19).

Figure 19- Samples handling.

1.2. Chemical characterization of lipophilic fraction

To determine the lipophilic profile of *S. ramosissima,* the four samples harvested were selected. The plants were freeze-dried and ground prior to extraction according to a previously reported methodology (109). Each sample was submitted to a Soxhlet extraction with dichloromethane for 6 h and the solvent was evaporated to dryness at low pressure. The dried extracts were weighed and the results are expressed as percentages of dry biomass material.

Gas chromatography-mass spectrometry (GC-MS) analysis

Before GC-MS analysis, nearly 20 mg of each dried sample was converted into trimethylsilyl (TMS) derivatives according to a previously optimized methodology (109). Each sample was dissolved in 150 μL of pyridine, 100 μL of tetracosane solution dissolved in pyridine, 4 mg/mL (internal standard), and compounds with hydroxyl and carboxyl groups were converted into TMS ethers and esters, respectively, by adding 250 μL of *N,O*-bis(trimethylsilyl)-trifluoroacetamide and 50 μL of trimethylchlorosilane. The mixture was maintained at 70 °C for 30 min.

GC-MS analyses were performed using a Trace gas chromatograph (2000 series) equipped with a Thermo Scientific DSQ II mass spectrometer (Waltham, MA). Separation of compounds was carried out in a DB-1 J&W capillary column (30 m × 0.32 mm inner diameter, 0.25 μm film thickness) using helium as the carrier gas (35 $cm.s^{-1}$). The chromatographic conditions were as follows: initial temperature, 80°C for 5 min;

temperature rate, 4°C min^{-1} up to 260°C, 2 °C min^{-1} up to 285°C, which was maintained for 8 min; injector temperature, 250 °C; transfer-line temperature, 290 °C; split ratio, 1:50.

The mass spectrometer was operated in the electron impact (EI) mode with an energy of 70 eV, and data were collected at a rate of 1 $scan.s^{-1}$ over a range of mass-to-charge ratio (*m/z*) 33-700. The ion source was kept at 250 °C. Chromatographic peaks were identified by comparing their mass spectra with the equipment mass spectral library (Wiley-NIST Mass Spectral Library, 1999) and with literature data (as specifically addressed in the Results and Discussion section) and, when needed, by injection of standard samples. In some cases, identification was also confirmed based on characteristic retention times (RTs) under the described experimental conditions.

In order to estimate the content of each compound, the chromatographic areas were extracted. The area and concentration of the internal standard was used to express the concentration of the compounds as internal standard equivalents. The compound contents were expressed as mg/100 g of dw of plant biomass, mean values ± relative standard deviation.

A data matrix consisting of 35 variables (metabolites) and 12 observations was constructed (Table 9). The 12 observations correspond to 4 samples (each one with 3 independent aliquots), on the global content or on the content of each chemical family under study. One-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD) using the GraphPad Prism version 6 for Windows (trial version, GraphPad Software, San Diego California, USA) was performed. It was considered statistically significant when $p < 0.01$.

1.3. Chemical characterization of polar fraction

To determine the phenolic profile of *S. ramosissima*, the plants were freeze-dried and ground prior to extraction (106). Each sample (5g) was submitted to a methanolic extraction for 12h and the extraction occurred in the absence of light and under constant stirring, at room temperature. The methanol extract was filtered under vacuum and evaporated on a rotary evaporator at 30 ºC. The plant extracts were dissolved in hot, distilled water and a liquid-liquid extraction of the non-polar phase was performed with petroleum ether. The aqueous extracts were evaporated on rotary evaporator at 30°C and dissolved in ethanol/ distilled water (50:50), being 200 mg/mL, the final concentration of working solution.

Total phenolic content determination

The total phenolic content (TPC) of the samples was determined by the Folin-Ciocalteu method as previous described (110), with some modifications. The reagents used in this method were sodium carbonate (106 mg. mL^{-1}), Folin-Ciocalteu's reagent (1:1 in water), ethanol/ distilled water (50:50) and distil water. The method is based on the addition of 200 μL of water, 15 μL of sodium carbonate, 15 μL of the sample (extract) and 15 μL of Folin-Ciocalteu's reagent. For the blank, the same amounts of sodium carbonate, Folin-Ciocalteu's reagent and water were pipped and 15 μL of methanol was pipped instead of the sample. The solutions were kept for 30 min in the absence of light. All the measurements were made in triplicate, using three aliquots of each extract and the average value was calculated in each case. The assay was performed in a 96-well microplate using a BioTek ® microplate reader (at 760 nm). The calibration curve was performed with 6 gallic acid solutions with concentration range between 0.0625 – 1.0 mg/mL. The results were expressed as mg of GAE/100 g fresh weight and dry weight. Determination of selected phenolics in extracts *S. ramosissima* was conducted according to a previously methodology (111). The analysis was performed at Department of Chemistry, Biochemistry and Environmental Protection, University of Novi Sad, Serbia.

Liquid chromatography – mass spectrometry (LC-MS) analysis

Briefly, samples and 45 standards (prepared in serial dilutions, ranging from 1.53 ng/mL to $25.0x10^3$ ng/mL, dissolved in a mixture of 0.5% formic acid and methanol (in 1:1 ratio)) were analysed using Agilent Technologies 1200 Series HPLC coupled with Agilent Technologies 6410A QqQ mass spectrometer with electrospray ion source and controlled by Agilent Technologies Mass Hunter Workstation software (ver. B.03.01). Injection volume was 5 μL.

Separation was performed using Zorbax Eclipse XDB-C18 (Agilent Technologies) column, 50 mm x 4.6 mm, 1.8 μm, held at 50 °C. Mobile phase, consisting of 0.05% aqueous formic acid (phase A) and methanol (B) was delivered at flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, post time 3 min). Ion source parameters were: nebulization gas pressure 40 psi, drying gas flow 9 L/min and temperature 350 °C, capillary voltage 4000 V. All compounds were detected in negative mode, using dynamic selected reaction monitoring with optimized compound-specific parameters (retention time, precursor ion, product ion, fragmentor voltage, collision voltage). Concentrations of standard compounds in extracts were

determined from the peak areas by using the equation for linear regression obtained from the calibration curves (Figure 28, Appendix 1).

1.4. Mineral content

The analysis was requested to Laboratório Central de Análises (LCA) from Aveiro University. Approximately 200 mg each dry sample were microwave digested (CEM, Mars 5), according to a previously optimized methodology (112), in high-pressure Teflon vessels (XP-1500, FEP) with 3 mL HNO_3, at 170°C and in closed system. Na, K, Mg and Se elements were by Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES, Horiba Jobyn Yvon, Model: Activa M), while Pb and Hg elements were analysed by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS, Thermo, Model: X series 2). For the minerals quantification, calibration curves were performed. Results displayed the limit of quantification (LOQ) and limit of detection (LOD).

2. Results and Discussion

2.1. Lipophilic profile

For GC-MS analysis, the plants of the three different locations described before were used. The dichloromethane extraction yields (Table 8) of the *S. ramosissima* samples ranged between 2.35% and 7.92% and Santiago da Fonte location presented higher yield.

Table 8- Dichloromethane extraction yields (% W/W) for *S. ramosissima* harvest from three different locations (dry weight).

Location	**Extraction yield (% w/w)** (n= 3)	RSD*
MP	2.36	0.85
MPR	3.40	16.6
BC	2.35	7.8
SF	7.92	1.3

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte;
* Relative standard deviation expressed in %

The lipophilic fraction of *S. ramosissima* harvested from three different locations presented quantitative differences in chemical composition between the different extracts.

The chromatogram of the derivatized dichloromethane of BC extract is illustrated in Figure 20. The chromatograms for the others extracts (SF, MP and MPR) are illustrated in Figure 29 (Appendix 2).

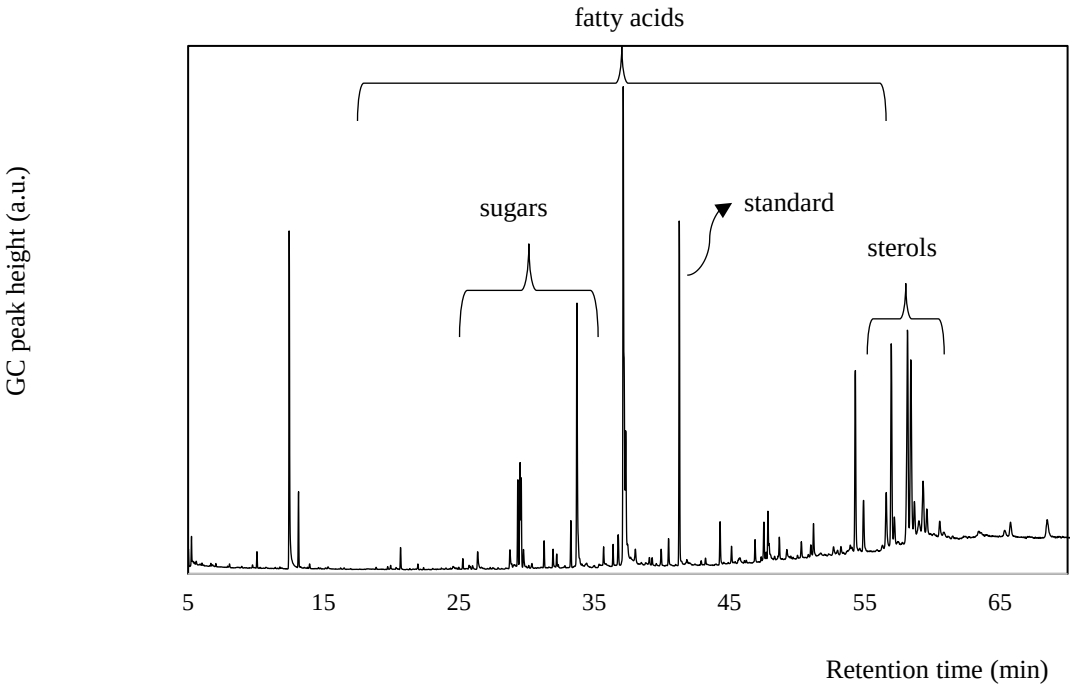

Figure 20- GC-MS chromatogram of the TMS-derivatized dichloromethane extract of *S. ramosissima* plant harvested in Boco river (BC), Aveiro, Portugal.

Figure 21 shows the families of compounds identified in the plant and dichloromethane extract of *S. ramosissima*.

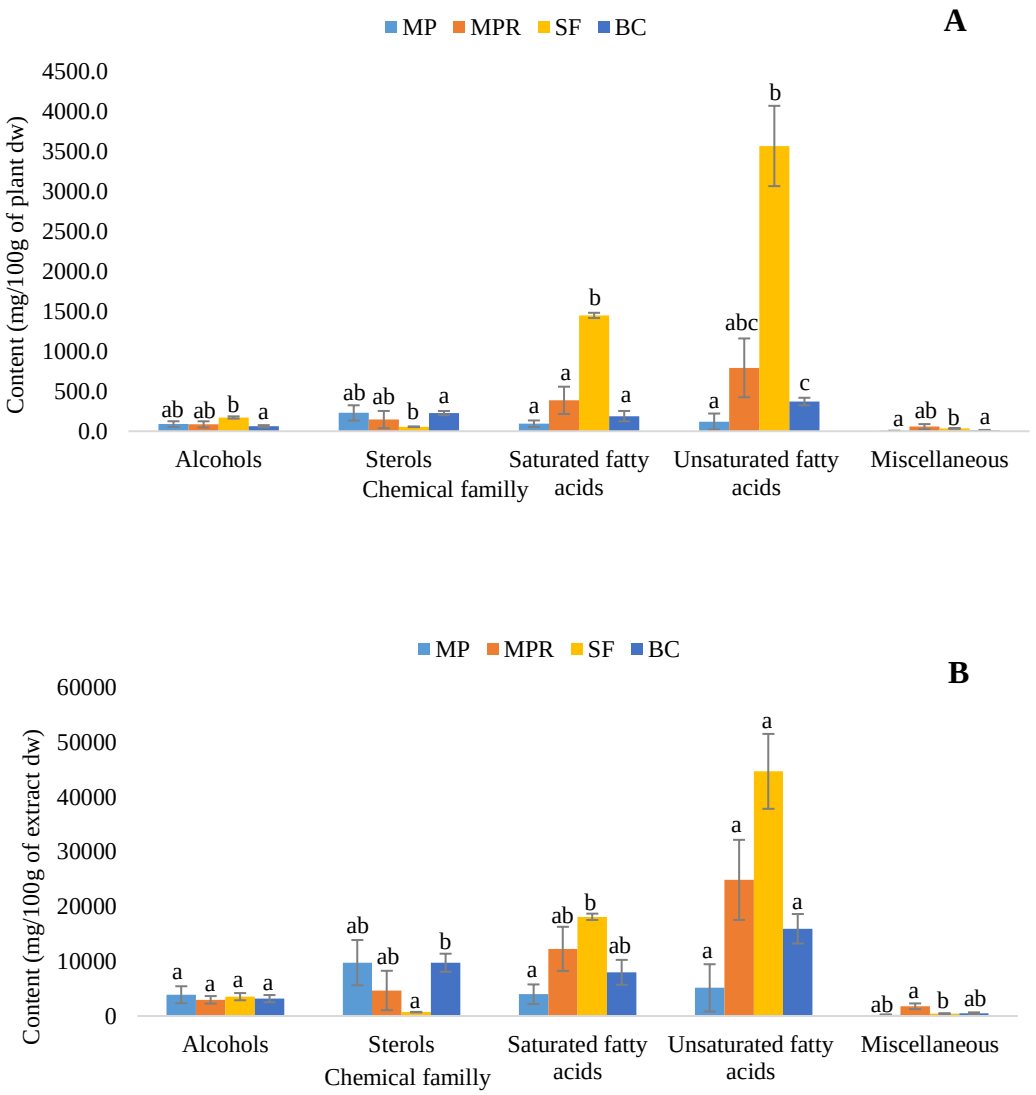

Figure 21- Lipophilic families in glasswort, harvested at three locations from Ria de Aveiro, Portugal. Mean values with unlike letters in different families show statistically significant differences ($p < 0.01$, Tukey's test). A – Plant; B – Extracts;

Fatty acids are the main family of compounds identified in the dichloromethane extract of *S. ramosissima* in all four samples. SF is the sample with higher content of saturated and unsaturated fatty acids but with lower content of sterols, being MP and BC, the samples with higher sterols content.

Table 9 shows the different families and individual compounds identified in the dichloromethane extracts, as well as their quantification in the four samples. The different families of lipophilic components present on *S. ramosissima* will be described below.

Table 9- Composition (mg/100g) of dichloromethane extracts from glasswort, harvested at three locations from Ria de Aveiro lagoon, Portugal.

Peak	R.t. (min)	Coumpound	MP	MPR	SF	BC
Content (mg/100g of extract) and RSD (%) **n=3 (results express in tetracosane equivalents)**						
Saturated fatty acids						
3	15.13	Nonanoic acid (C9:0)	69.6 (48)	41.8 (38)	36.0 (51)	21.6 (32)
4	29.11	Tetradecanoic acid (C14:0)	145.6 (55)	67.5 (44)	133.2 (19)	37.1 (18)
5	31.55	Pentadecanoic acid (15:0)	27.9 (23)	113.5 (44)	55.8 (18)	59.3 (26)
8	33.74	Hexadecanoic acid (C16:0)[1]	1397.7 (73)	7275.4 (29)	12635.0 (9)	3974.6 (23)
10	35.71	Heptadecanoic acid (C17:0)	324.2 (18)	274.7 (39)	20.8 (20)	131.4 (25)
15	38.06	Octadecanoic acid (C18:0)[1]	233.5 (41)	1356.3 (56)	2312.7 (2)	519.0 (47)
17	42.00	Eicosanoic acid (C20:0)	26.3 (37)	424.4 (26)	1948.6 (16)	110.6 (21)
19	44.33	Heneicosanoic acid (C21:0)	401.5 (32)	538.0 (47)	220.9 (51)	498.4 (10)
21	45.79	Docosanoic acid (C22:0)	29.4 (35)	716.8 (33)	293.6 (21)	233.1 (47)
24	49.28	Tetracosanoic acid (C24:0)	165.5 (57)	761.5 (43)	224.2 (33)	285.6 (53)
28	54.93	Hexacosanoic acid (C26:0)	329.0 (28)	558.8 (47)	50.8 (20)	755.5 (27)
29	56.63	Octacosanoic acid (C28:0)	305.4 (31)	124.5 (49)	178.2 (44)	1340.9 (49)
	Subtotal/ Subtotal (mg/ 100g dw of plant)		**3455.6 / 93.8**	**12253.2 / 386**	**18073.8 / 1449**	**7945.5 / 189**
Unsaturated fatty acids						
12	37.18	9,12-Octadecadienoic acid (C18:2) + 6,9,12 - octadecatrienoic acid(C18:3)	4610.7 (88)	18810.0 (30)	33975.9 (16)	14139.2 (21)
13	37.30	Octadec-11-enoic acid (C18:1)	357.1 (65)	5815.3 (28)	10352.3 (13)	1684.2 (28)
14	37.50	Octadec-9-enoic acid (C18:1)	169.0 (42)	222.5 (44)	328.9 (7)	99.8 (55)
	Subtotal/ Subtotal (mg/ 100g dw of plant)		**5136.8 / 120**	**24847.8 / 793**	**44657.1 / 3570**	**15923.2 / 372**
Alcohols						
2	13.16	Glicerol	140.9 (47)	580.9 (40)	1611.7 (35)	744.1 (28)
6	31.98	Hexadecan-1-ol (C16:0)	256.4 (32)	313.6 (36)	4.59 (54)	174.3 (22)
7	33.58	Heptadecan-1-ol (C17:0)	3.88 (33)	125.0 (66)	12.7 (26)	12.3 (79)

Peak	R.t. (min)	Coumpound	MP	MPR	SF	BC
11	36.41	Octadecan-1-ol (C18:0)	228.2 (39)	460.6 (51)	10.2 (19)	152.4 (36)
16	40.50	Eicosan-1-ol (C20:0)	127.7 (39)	314.4 (50)	277.7 (19)	221.9 (22)
20	44.47	Docosan -1- ol (C22:0)	33.7 (30)	251.2 (24)	215.3 (21)	141.9 (7)
22	46.11	Tricosanol (C23:0)	23.9 (10)	48.5 (53)	14.0 (35)	51.5 (24)
23	47.85	Tetracosanol (C24:0)	830.9 (47)	277.6 (13)	1488.3 (4)	973.6 (30)
25	49.55	Pentacosanol (C25:0)	16.7 (6)	58.3 (45)	10.2 (21)	14.4 (32)
26	51.22	Hexacosanol (C26:0)	241.0 (31)	393.6 (71)	79.0 (10)	573.1 (32)
27	54.60	Octacosanol (C28:0)	1842.3 (62)	371.1 (1)	19.1 (29)	32.6(20)
34	58.98	Triacontanol (C30:0)	189.8 (26)	29.1 (54)	15.4 (45)	265.3 (33)
	Subtotal/ Subtotal (mg/ 100g dw of plant)		**3935.4 / 92.8**	**3223.9 / 104**	**3758.2 / 302**	**3357.4 / 79.3**
Miscellaneous						
1	11.45	Butanedioic acid (C4:0)	28.6 (40)	705.6 (43)	125.7 (32)	39.4 (29)
9	34.37	Isoferulic acid	87.5 (68)	974.4 (29)	18.7 (14)	401.1 (44)
18	43.24	Pentacosane	90.5 (16)	92.7 (21)	293.4 (11)	62.5 (14)
	Subtotal/ Subtotal (mg/ 100g dw of plant)		**206.6 / 4.99**	**1772.7 / 57.7**	**437.8 / 35.1**	**503.0 / 12.0**
Sterols						
30	57.18	24-Etil-δ(22)-coprostenol	195.1 (42)	451.1 (38)	30.8 (28)	362.8 (16)
31	56.99	Stigmasterol[1]	2229.7 (41)	1526.3 (76)	186.3 (13)	3030.9 (14)
32	58.19	β-Sitosterol[1]	4303.0 (54)	1381.1 (99)	295.9 (5)	3102.2 (15)
33	58.40	Sitostanol	1817.1 (38)	1100.1 (75)	119.1 (13)	2500.0 (16)
35	59.30	Stigmast-7-en-3-ol	1187.6 (27)	195.5 (63)	68.1 (18)	726.8 (44)
	Subtotal/ Subtotal (mg/ 100g dw of plant)		**9732.5 / 229**	**4654.1 / 148**	**700.2 / 56.1**	**9722.7 / 227**
		Total	**22466.9**	**46751.7**	**67627.1**	**37451.8**
		Total (mg/ 100g dw of plant)	**541**	**1500**	**5412**	**879**

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte; [1]Identification by co-injection of standards;
RSD - Relative standard deviation expressed in %;

Fatty acids

Fatty acids were identified as TMS derivatives based on their fragmentation pattern and elution order. The most abundant peaks in the mass spectra are those at *m/z* 73 and 75 and the $[M\text{-}CH_3]^+$ ion (or $[M\text{-}15]^+$). The ions at *m/z* 73 $[(CH_3)_3Si]^+$ and 75 $[(CH_3)_2Si\text{-}OH]^+$ provide negligible or no structural information since they are ubiquitous in the mass spectra of all TMS derivatives. Other prominent peaks appear at *m/z* 117, 129, 132, 145. Ions at *m/z* 132 and 145 are McLafferty type rearrangement ions. The ions referred are visible, for example, in the mass spectrum of the TMS derivative of hexadecanoic acid shown in Figure 22.

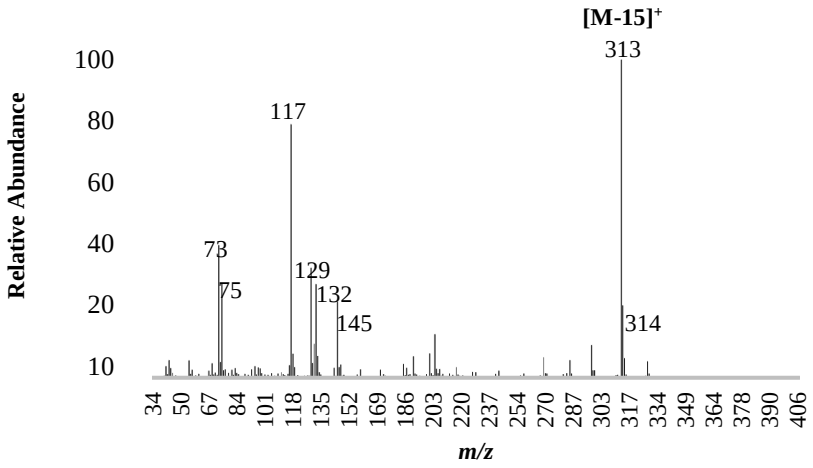

Figure 22 - Mass spectrum of the TMS derivative of hexadecanoic acid.

Fatty acids represented one of the main groups of lipophilic compounds present in *S. ramosissima* plant corresponding to 38.2-95.7% of the total amount of detected compounds in dichloromethane extract. Both saturated fatty acids and unsaturated fatty acids were identified in *S. ramosissima* plants. The content of unsaturated fatty acids was found higher than the content of saturated fatty acids in all samples. The identified fatty acids ranged from nonanoic acid (C9:0) to octacosanoic acid (C28:0), including four unsaturated structures (C16 and C18). Palmitic acid (C16:0) was the most abundant saturated fatty acid in *S. ramosissima* plant with the highest content observed in Santiago da Fonte extract (SF) (126.35 mg/g of extract) and the lowest in Marinha dos Peixinhos extract (MP) (13.98 mg/g of extract). Linoleic and linolenic acids were the major compounds of the unsaturated fatty acids group with the highest content detected in Santiago da Fonte, reaching 339.76 mg/g of extract.

Alcohols

Alcohols were identified as TMS derivatives based on their fragmentation pattern and elution order. The most abundant peaks in the mass spectra are *m/z* 73 and 75 and the ion $[M-15]^+$. Also, there are other prominent peaks that appear at *m/z* 89 and 103 $[(CH_3)_3SiOCH_2]^+$ and help to distinguish aliphatic alcohols from fatty acids. The ions referred are visible in the mass spectrum of the TMS derivative of octadecan-1-ol shown in Figure 23.

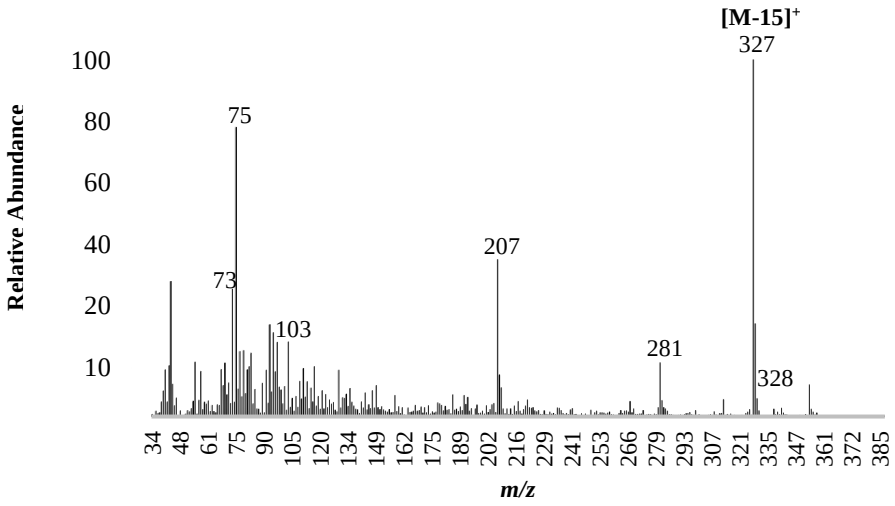

Figure 23- Mass spectrum of the TMS derivative of octadecan-1-ol.

Alcohols were detected in quite low amounts, representing about 5.6-17.5% of the total lipophilic extractives of the different samples of *S. ramosissima*. Tetracosanol and octadecan-1-ol were the most abundant compounds in Marinha dos Peixinhos sample, representing 6.61 – 39.6 % of the total alcohols present, followed by hexadecan-1-ol, glycerol and Docosan-1-ol.

Sterols

In general, the mass spectra of sterols exhibit the presence of abundant TMS-containing groups because this strongly directs the fragmentation of the molecules. The ions at *m/z* 73 and 75 provide no structural information and correspond to the loss of $(CH_3)_3Si$ and $(CH_3)_2Si\text{-}OH$ respectively. Ions at *m/z* 129 and $[M-129]^+$ correspond to the loss of the TMS group together with a carbon fragment of ring A containing the C-1, C-2 and C-3. Another important structural ion is at *m/z* $[M-90]^+$ which corresponds to the 1,2-elimination of the trimethylsilanol group. Indirect information about the molecular

weight can be obtained from *m/z* $[M-15]^+$. The ions referred to above and some fragmentations are visible in the mass spectrum of the TMS-derivative of β-sitosterol shown in Figure 24.

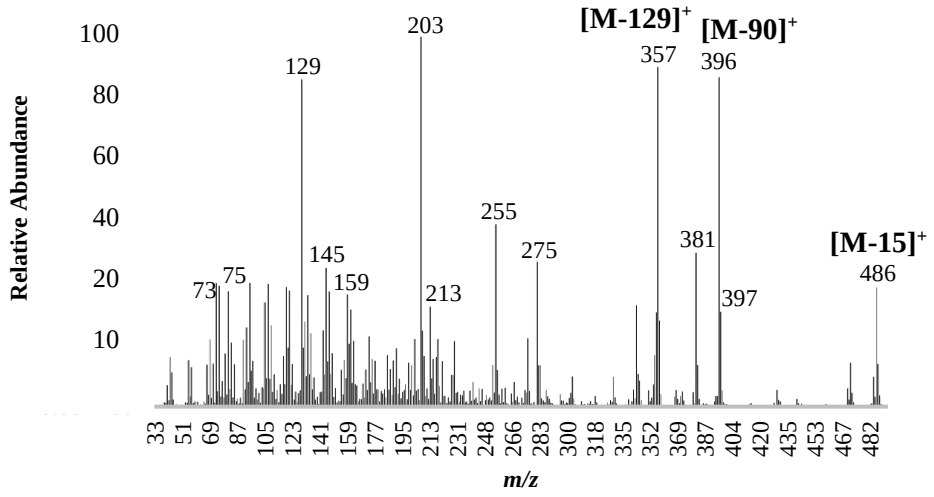

Figure 24- Mass spectrum of TMS derivative of β-sitosterol.

The sterols found in *S. ramosissima* plants extract were 24-Etil-δ(22)-coprostenol, stigmasterol, β-sitosterol, sitostanol and stigmast-7-en-3-ol. These phytosterols represents 1.0-43.3 % of the total extract. β-sitosterol and stigmasterol were the main components of this family in all samples representing 31.9-44.2% and 22.9-42.3% of total sterols content, respectively. All the sterols identified in this work were already reported in the literature for *S. ramosissima* species (20).The role of phytosterols in human health is mainly associated with non-pharmacological lipid-altering therapies, reducing exogenous cholesterol absorption by displacement in the intestinal lumen. Thus, both total cholesterol and HDL are reduced. Consumption of 1.8-2.0 g of phytosterols per day has been shown to lower cholesterol absorption.

Miscellaneous

The other three compounds identified belongs to different chemical families, alkanes, diacids and phenolic acids. These compounds were not present in high levels in *Salicornia* samples, being MPR, the sample with higher content when compare to the other three samples analysed. Isoferulic acid is the phenolic acid identified by this technique and it presents in 0.03 – 2.1% of total compounds detected. MPR is the sample with higher percentage of this compound (2.1%) that corresponds to 31.3±8.31 mg/100g

of plant express in dry weight. The lipophilic profile of *S. ramosissima* samples was quite different when compared with previous results (20).

The reported lipophilic profile of this halophyte were manly alcohols and fatty acids. However, palmitic acid was the most abundant compound reported before, followed by tetracosanol. The present results showed quantitative differences, which may be related to the harvest period, since, these results were obtained for the fructification stage and the previous results were reported for the vegetative stage of this species. Although all identified compounds were identified before, this analysis showed higher amounts of lipophilic compounds, manly fatty acids and sterols.

2.2. Phenolic profile

The extraction yield of *S. ramosissima* obtained with methanol was 32.2, 29.2, 28.6 and 19.5% for MP, MPR, BC and SF, respectively. The total phenolic content was evaluated by Folin-Ciocalteu method and an overall view of these content for *S. ramosissima* samples are shown in Table 10.

Table 10- Total phenolic content for glasswort, harvested at three locations from Ria de Aveiro lagoon, Portugal.

Samples	Total phenolics (mg GAE/100g dw)
S. ramosissima	
MP	2733.9 ± 62.9[a]
MPR	3397.5 ± 96.4[b]
SF	1391.3 ± 123.2[c]
BC	1747.4 ± 125.4[d]

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte;
Values expressed as mean ± SD; Mean values with unlike letters in columns show statistically significant differences ($p < 0.01$, Tukey's test);
GAE – gallic acid equivalents;
dw – dry weight

The total phenolic content (TPC) for *S. ramosissima* samples is significantly different ($p<0.01$), MPR is the sample that presents higher phenolic content (3398 mg gallic acid equivalents /100g dry weight) followed by MP sample (2734 mg/ gallic acid 100g dry weight. The results obtained in MP and MPR samples are in accordance with previous studies (Table 3). SF and BC samples present a lower value when compared

with previous results and when compared with MP and MPR samples (Table 3). However, all the results are in concordance with previous studies when compared with other *Salicornia* species since the values for TPC ranged between 890 - 3309 mg GAE/ 100g of dw (Table 3). The results aim that *S. ramosissima* present higher phenolic content than all the others *Salicornia* species (Table 3).

LC-QqQ-MS analysis

Identification of 31 phenolic compounds in *Salicornia ramosissima* extracts was performed using LC-MS. The phenolic compounds were quantified using calibration curves of standard compounds (Appendix 3, Table 19). The identification of the selected phenolic compounds is shown in Table 11. The results showed that MP and MPR are the samples with more compounds identified as well as some differences between the four *S. ramosissima* extract.

Table 11- Identification of detected phenolics in *S. ramosissima* polar extract.

Compound[1]	**Content of selected phenolics (μg/g of dw)** *S. ramosissima* **extracts**			
	MP	MPR	BC	SF
Non-flavonoids				
p-Hydroxybenzoic acid	<LOQ	15.9	<LOQ	9.5
Gentisic acid*	<LOQ	<LOQ	<LOQ	<LOQ
Protocatechuic acid*	1.54	2.54	2.30	3.83
p-Coumaric acid*	2.68	6.64	0.16	0.16
Escutelin*	<LOQ	<LOD	___	<LOD
Isoscopoletin*	4.4	24.6	2.3	___
Scopoletin*	1.68	10.8	<LOD	___
Ellagic acid*	<LOD	<LOD	<LOD	<LOD
5-*O*-Caffeoylquinic acid	145.9	50.1	34.4	___
Quinic acid*	3.59	5.68	2.48	1.09
Caffeic acid	11.4	11.8	7.50	___
Ferulic acid*	8.46	18.0	4.02	2.90
Vanillic acid*	<LOD	<LOD	___	<LOD
Cinnamic acid*	___	<LOD	___	<LOD
Secoisolaricisesinol*	___	<LOD	___	___
Syringic acid	___	<LOD	<LOD	<LOD
Flavonoids				
Kaempferol-3-*O*-glucoside*	<LOQ	<LOQ	<LOQ	<LOD

Compound[1]	**Content of selected phenolics (μg/g of dw)**			
	***S. ramosissima* extracts**			
	MP	MPR	BC	SF
Baicalein*	___	___	___	0.37
Chrysoeriol*	0.29	0.38	0.17	0.28
Kaempferol*	<LOQ	59.0	<LOQ	<LOQ
Isorhamnetin-3-*O*-glucoside + Quercetin-3-*O*-galactoside	153.7	93.1	31.5	<LOD
Isorhamnetin	680.5	1402.4	<LOD	___
Rutin*	<LOQ	<LOQ	<LOD	___
Diosmetin*	3.0	1.0	___	1.0
Luteolin 7-*O*-glucuside*	<LOQ	<LOQ	<LOD	___
Amentoflavone*	<LOD	___	___	___
Isoliquiritigenin*	<LOD	<LOD	___	___
Pinostrobin*	<LOD	___	___	___
Quercetin	<LOD	<LOD	___	___
Total	**1012.9**	**1676.6**	**82.4**	**5.6**

MP- Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC- Boco river; SF – Santiago da Fonte;
LOD: limit of detection; LOQ: limit of quantification; *first reported in *S. ramosissima* species;
[1]Identification by co-injection of standards;

Presence of caffeic acid, caffeoylquinic acid, isorhamnetin glucoside and isorhamnetin was already reported in *S. ramosissima* species (51). However, to the best of our knowledge a total of 22 compounds were reported for the first time as *S. ramosissima* components, namely gentisic acid, protocatechuic acid, p-coumaric acid, escutelin, isoscopoletin, ellagic acid, quinic acid, ferulic acid, vanillic acid, cinnamic acid, secoisolaricisesinol, kaempferol-3-*O*-glucoside, baicalein, chrysoeriol, kaempferol, rutin, diosmetin, luteolin-7-*O*-glucoside, amentoflavone, isoliquiritegin and pinostrobin. Although some of these compounds, have already been reported as constituents of other *Salicornia* species, namely protocatechuic acid, ferulic acid, p-coumaric acid, cinnamic acid, rutin, among others (Table 3), being p-coumaric acid, the only compound quantified (*S. freitagii*) (54). Among the phenolic compounds identified for the first time in *S. ramosissima*, three were reported for the first time as components of the *Salicornia* genus, namely escutelin, ellagic acid and isoscopoletin.

The phenolic content of each extract was estimated, caffeic acid, 5-*O*-caffeoylquinic acid, ferulic acid, isoscopoletin, isorhamnetin-3-*O*-glucoside/quercetin-3-*O*-galactoside, protocatechuic acid, p-coumaric acid, scopoletin, quinic acid, baicalein, chrysoeriol, kaempferol, isorhamnetin and diosmetin were the phenolic compounds

quantified. However, the concentration of these compounds was not estimated in all extracts of *S. ramosissima*. Caffeic acid, 5-*O*-caffeoylquinic acid, isorhamnetin-3-*O*-glucoside/quercetin-3-*O*-galactoside, and isoscopoletin were estimated in MP, MPR and BC extracts, scopoletin as well as isorhamnetin were estimated only in MP and MPR extracts and baicalein was estimated only in SF extract. To the best of our knowledge the estimated content of these compounds was reported for the first time in this present analysis for *Salicornia* genus. Isorhamnetin, isorhamnetin 3-*O*- glucoside/quercetin 3-*O* galactoside and 5-O-caffeoylquinic acid were the main components. The phenolic content of each extract is shown in Table 11, expressed in μg/g of extract. The MP and MPR extracts were found to have higher amount of phenolic compounds (1012.9 and 1676.6 μg/g, respectively). The results obtained were lower than the total phenolic content obtained in Table 10, since, the method for total phenolic content is not specific for phenolic compounds. However, MP and MPR samples showed also higher amount of total phenolic content (Table 10), being in accordance with the results obtained for LC-QqQ-MS assay.

These results allow verification of the MP and MPR extracts as the most promising to extract phenolic compounds. These methodology (LC-QqQ-MS) allowed to identify a panoply of non-reported phenolic compounds for *S. ramosissima* species, which is an important basis for further research and for adding value to this halophyte, considering it as a food with health benefits.

2.3. Mineral content

The mineral content of the studied species is shown in Table 12. The essential minerals (Na, K, Mg, Se) and toxic compounds (Pb, Hg) were quantified by ICP-OES and ICP-MS, respectively.

Table 12- Essential minerals and toxic compounds of *S. ramosissima* samples express in dw and fw of plant.

Minerals	Content (dry weight)						Content (fresh weight)					
	Se (µg/g)	**K (mg/g)**	**Mg (mg/g)**	**Na (mg/g)**	**Pb (µg/g)**	**Hg (µg/g)**	**Se (µg/g)**	**K (mg/g)**	**Mg (mg/g)**	**Na (mg/g)**	**Pb (µg/g)**	**Hg (µg/g)**
S. ramosissima												
MP	1.4	16	6.3	95	<1.8*	<0.013*	0.26	3.0	1.2	17.9	____	____
MPR	1.2	18	7.9	105	<1.8*	<0.013*	0.21	3.2	1.4	18.6	____	____
SF	1.6	10	8.8	104	<1.8*	<0.013*	0.29	1.8	1.6	21.2	____	____
BC	1.4	13	11	123	<1.8*	<0.013*	0.24	2.2	1.9	18.8	____	____
					RDI		55.0	3500	450	1500	_	_
					Limit levels in food		_	_	_	_	0.2	0.5

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte;
RDI – Recommended daily intake;
dw – dry weight;
fw – fresh weight;
*LOQ: limit of quantification; LOD (limit of detection): <0.58 and <0.0039 for Pb and Hg, respectively;

S. ramosissima showed a range between 95 – 123 mg/g for sodium, 10-18 mg/g for potassium, 6.3-11 mg/g for magnesium and 1.2-1.4 μg/g for selenium. Potassium, magnesium and sodium levels were similar to previous studies for *Salicornia ramosissima* and they were also similar to other edible wild plants (5).

These four minerals are essential to the organism and there are a recommended daily intake (RDI) for them. In order to evaluate the necessary amount of S. ramosissima and salt to obtain the recommended daily intake, some calculations were performed and the results are showed in Table 13.

Table 13 - Necessary amount of *S. ramosissima* and salt to obtain the recommended daily intake (RDI) for the 4 essential minerals.

Mineral	**RDI**[1]	***Salicornia ramosissima***	**Salt**
Sodium (Na)	1500 mg	70.9 – 83.9 g fresh weight 12.2 – 15.8 g dry weight	3.75 g
Potassium (K)	4700 mg	1468.8 – 2006.7 g fresh weight 261.1 – 470.0 g dry weight	____
Magnesium (Mg)	450 mg	236.8 – 375.0 g fresh weight 40.9 – 71.4 g dry weight	15 g
Selenium (Se)	55 μg	189.7 – 261.9 fresh weight 34.4 – 45.8 g dry weight	____

[1]Reference (60)

These results showed that this halophyte can be a source of important minerals such potassium, magnesium and selenium. However, it is necessary high amounts of *S. ramosissima* to obtain the recommended daily intake for these minerals. On the other hand, a consumption of 3.75 g of salt corresponds to the recommended daily intake for sodium. *S. ramosissima* appears to be a good alternative to common salt, since a

consumption of 100g (fresh plant) represents a sodium intake range between 1786 - 2116 mg (Table 12). These range values for sodium are obtained with just 5g of salt, which corresponds to the recommended maximum daily intake for salt (85).

Besides this, halophytes can be exposed to heavy metals in polluted zones as previous reported (68). In order to evaluate the presence of toxic compounds in this species, lead and mercury contents were analysed, being the obtained levels below the limit of quantification (LOQ), 1.8 µg/g and a 0.013 µg/g, respectively. Since, the values for these two toxic compounds (Hg and Pb) were below the LOQ, the conversion to fresh weight to compare with the legislated values was not possible (0.5µg – 1.0 fresh weight for Hg and 0.2µg/g for Pb) (113).

Chapter III – Evaluation of biological effects of *S. ramosissima*: Antioxidant and anti-inflammatory activity

Objectives

Nowadays, the search for natural antioxidants sources that may contain biological effects in human diet is increasing. Antioxidant activity is one of the biological activities reported in this species (51,54), however this biological activity is not detailed reported for this species. Furthermore, anti-inflammatory activity of *S. ramosissima* was also explored, since there is a growing interest in natural alternatives that can act in anti-inflammatory process. There are some *in vitro* and *in vivo* studies for this genus (Table 5), however there are not enough studies about their related bioactivities.

1. Materials and Methods

This chapter follows the same experimental design as showed in chapter II, being the extracts from polar fraction (methanol extraction) used to evaluate *in vitro* antioxidant and anti-inflammatory activity (samples extraction described in Chapter II). The anti-inflammatory and antioxidant activity was evaluated at Department of Chemistry, Biochemistry and Environmental Protection, University of Novi Sad, Serbia

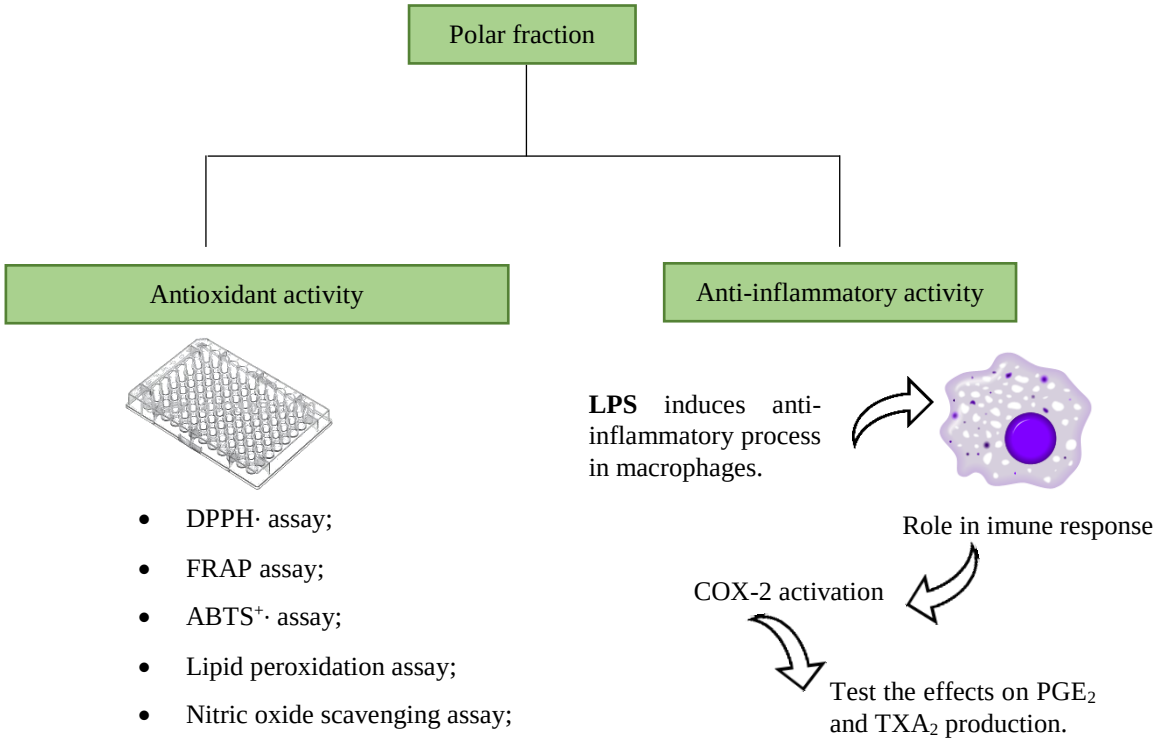

Figure 25- Experimental design of the work developed in this chapter. LPS – lipopolysaccharide; PGE_2 – prostaglandin E_2; TXA_2 – thromboxane A_2;

1.1. Antioxidant activity determination

The antioxidant activity and radical scavenging radicals were evaluated by lipid peroxidation, reducing power (FRAP) assay, NO scavenger capacity, reduction of DPPH· radical and reduction of $ABTS^{+}\cdot$ cation radical. The correction means the correction of the extract colour.

Reduction of DPPH· Radical

The radical scavenging activity was determined by the DPPH· method according to previous methodology (95), with some modifications. The reagents used in this method was methanol, 0.394 mg/mL DPPH solution in methanol and ethanol/ distilled water (50:50). For the blank, 201.6 µL of methanol and 24 µL of a 0.394 mg/mL DPPH solution in methanol were pipped into each well. For each sample analysis, 9.6 µL of the extract, 24 µL of DPPH solution and 192 µL of methanol were pipped into the wells. Each extract was analysed in triplicate.

Absorbance of extracts and control was measured at 514 nm, with a BioTek ® microplate reader by using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. The radical scavenging activity was expressed in IC_{50}, whereas the % of scavenging rate was calculated using the formulae:

$$\%\ \boldsymbol{scavenging} = \frac{Abs\ (Control) - Abs(Extract)}{Abs\ (Control)} * 100$$

Reducing Power (FRAP) Assay

To evaluate the reducing power of extracts, the ferric ion reducing antioxidant power (FRAP) assay based in previous methodology (104), modified for 96-well microplates was used. FRAP reagent was prepared by mixing 10 mM 2,4,6-tripyridyl-striazine (TPTZ) in 40 mM HCl, 0.02 M FeCl3, and acetate buffer, pH 3.6, in a ratio of 1:1:10, respectively. For the blank, 290 µL of FRAP reagent and 10 µL of solvent were pipped into each well. For each sample analysis, 10 µL of the extract and 290 µL of FRAP reagent were pipped into the wells and for the correction of the extract colour, 10 µL of the extract and 290 µL of distilled water were pipped into the wells. Each extract was analysed in triplicate. This assay was performed by measuring the absorbance at 593 nm after 6 min. The final absorbance (Abs) was calculated using the formulae:

$$\boldsymbol{Abs} = Extract\ Abs - (control\ Abs - correction\ Abs)$$

The reduce power was expressed as milligrams of ascorbic acid equivalents per mL of extract, a calibration curve was performed with 8 acid ascorbic solutions with concentration range between 10 – 160 µg/mL.

Lipid Peroxidation (Thiobarbituric acid assay)

To determinate the extent of Fe^{2+}/ascorbate-induced lipid peroxidation was determined by Thiobarbituric acid (TBA) assay (101), using polyunsaturated fatty acid (PUFA) as a substrate, obtained from linen semen by Soxhlet extraction. PUFA were added to phosphate buffer, pH 7.4, in the presence of 0.25% Tween-80 to obtain a 0.035% suspension. The reagents used in this method were 0.035 % emulsion of fatty acids (PUFA), ethanol/ distilled water (50:50), 4.58 mM $FeSO_4$, 0.087 mM ascorbic acid, 3.72 % EDTA and 1.0 M of TBA.This assay was performed by measuring the absorbance at 532 nm. For each sample and blank analysis, 1.5 mL of PUFA emulsion, 70 µL of the extract/solvent, 10 µL of $FeSO_4$ and 10 µL of ascorbic acid were added to a flask tube. For the correction of the extract colour, 1.52 mL of phosphate buffer and 70 µL of the extract were added to a flask tube, followed by incubation at 37 ºC for 60 min. After the incubation, 100 µL of 3.72 % EDTA and 1.0 of TBA reagent were added in all flask tubes and collocated to boil at 100 ºC. After these, the samples were cooled and centrifugated at 3500 o/min for 15 min. Each extract was analysed in triplicate.

The results were expressed in IC_{50}, whereas the % of scavenging rate was calculated using the formulae:

$$\%\ \boldsymbol{scavenging} = \frac{Control\ Abs - (Extract\ Abs - Correction\ Abs)}{Control\ Abs} * 100$$

Reduction of $ABTS^{+}\cdot$ cation radical

To evaluate the reducing of $ABTS^{+}\cdot$ cation radical of extracts, the ABTS assay, modified for 96-well microplates was used (97). For the blank, 290 µL of ABTS solution and 10 µL of solvent were pipped into each well. For each sample analysis, 10 µL of the extract and 290 µL of ABTS solution were pipped into the wells and for the correction of the extract colour, 10 µL of the extract and 290 µL of distilled water were pipped into the wells. Each extract was analysed in triplicate.

This assay was performed by measuring the absorbance at 734 nm after 6 min. The reduction of $ABTS^{+\cdot}$ cation radical was expressed in IC_{50}, whereas the % of scavenging rate was calculated using the formulae:

$$\%\ \boldsymbol{scavenging} = \frac{Control\ Abs - (Extract\ Abs - Correction\ Abs)}{Control\ Abs} * 100$$

NO Scavenging Capacity

The test of nitric oxide radical scavenging capacity was based on method previous published (114). The reagents used in this method were 10.0 mM solution of Sodium-nitroprusside dehydrate (SNP), ethanol/ distilled water (50:50), 0.067 M Phosphate buffer and Griess's reagent. Griess's reagent was prepare mixing equal amounts of sulfanilamide (2% in 4% phosphoric acid) and N-(1-naphthyl)ethylenediamine dihydrochloride (0.2%). For each sample and blank analysis, 10 µL of the extract/solvent, 75 µL of SNP and 75 µL of Phosphate buffer were added into the wells and for the correction of the extract colour, 10 µL of the working solution and 150 µL of Phosphate buffer were pipped into the wells. After 1h of incubation on constant light intensity, 150 µL Griess's reagent were added in all wells. Each extract was analysed in triplicate.

This assay was performed by measuring the absorbance at 546 nm after 1h. The results were expressed in IC_{50}, whereas the % of scavenging rate was calculated using the formulae:

$$\%\ \boldsymbol{scavenging} = \frac{\big(1 - (Extract\ Abs - Correction\ Abs)\big)}{Control\ Abs} * 100$$

1.4. Anti-inflammatory activity analysis

1.4.1. The effects on prostaglandin E_2 and thromboxane A_2 production in human leukemic U937 macrophages

The anti-inflammatory activity of *S. ramosissima* was studied in this present thesis by testing the PGE_2 and TXA_2 release from U937 macrophages. The four extracts were evaporated on rotary evaporator at 30°C and dissolved in ethanol/ distilled water (30:70) , being 300 mg/mL (106), the final concentration used in this analysis. The different steps of the procedure are described below.

1.4.2. Cell culture

Cell culture and transformation of cells was based in a previous methodology (115). U937 cells were maintained in suspension culture in RPMI-1640, supplemented with 10% (v/v) fetal bovine serum (FBS), 2.05 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B, at 37 °C, in a humidified atmosphere of 5% CO_2. The density of cells was kept between 2×10^5 and 2×10^6 cells/mL.

For differentiation into macrophages, 2×10^6 monocytes were seeded in 6-well plates containing 2 mL of complete culture medium and 100 nM phorbol 12-myristate 13-acetate (PMA). After 72 hours, supernatant was removed and fresh, serum-free medium was added.

1.4.3. Cytotoxicity evaluation

Monocytes (1×10^6 cells/mL) were cultured in serum-free medium containing tested compounds, solvent or equal amount of medium (control) for 22 hours. Morphological changes in the cells were observed microscopically, and the survival rates were evaluated by trypan blue exclusion 22 hours after treatments (116).

1.4.4. PGE_2 and TXA_2 release from U937 macrophages

Analysis of PGE_2 and TXA_2 release in U937 cells is based on modified methods (116,117). In brief, macrophages (2×10^6 cells, 2 mL of serum-free medium) were pre-treated with tested compounds (LT01-0516, LT02-0516, LT03-0516 and LT04-1016 in sub-toxic concentrations or solvent - DMSO) for 2 hours and stimulated with 0.5μg/mL LPS for 20 hours. Afterward, 1 μL of 20 mM arachidonic acid was added and cells were incubated at 37 °C with 5% CO_2 for 10 min. Acidification with cold 1% aqueous formic acid (0.4 mL) to pH 3 terminated the reaction. Extraction of products and internal standard was done according to previous procedure (106).

TXA_2 has a half-life of 30 seconds under physiological conditions and is converted to the stable metabolite TXB_2, so the levels of PGE_2, TXB_2 and internal standard PGB_2 were determined by LC-MS/MS, according to previously published procedure (106,118) with certain modification.

1.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD) using the GraphPad Prism version 6 for Windows (trial version,

GraphPad Software, San Diego California, USA) was performed. It was considered statistically significant when $p < 0.01$.

Pearson correlation coefficient was calculated in order highlight possible correlations using Microsoft Excel software 2016 (Microsoft, Redmond, WA, USA).

2. Results and Discussion

2.1. Antioxidant activity determination

Antioxidant supplements in food can contribute to the prevention of certain human diseases by reducing the amount of free radicals, being the reason to examine the antioxidant properties of *S. ramosissima*. The antioxidant activity can be manifested in a wide variety of actions, such as inhibition of oxidizing enzymes, chelation of transition metals, transfer of hydrogen or single electron to radicals (ROS), singlet oxygen deactivation, or enzymatic detoxification of ROS. Therefore, the total antioxidant activities should be evaluated through different methods to extensively characterize the antioxidant potential of the extracts. Thus, *S. ramosissima* extracts were examined regarding scavenging capacity toward DPPH, ABTS and nitric oxide radicals, ability to prevent lipid peroxidation, and reducing power (FRAP). Determined IC_{50} values (DPPH, Nitic oxide scavenging assay, Lipid peroxidation and ABTS – Appendix 3) and results for ferric reducing antioxidant power (FRAP) are shown in Table 14.

Table 14- Antioxidant activity of glasswort extracts, harvested at three locations from Ria de Aveiro lagoon, Portugal.

Extracts	**Antioxidant activity**				
	DPPH[1]	$^{\cdot}$NO[1]	LP[1]	ABTS[1]	FRAP[2]
S. ramosissima					
MP	1.85±0.02 [a]	1.07±0.05 [a]	5.55±0.21 [a]	0.308±0.013 [ab]	0.706±0.04 [a]
MPR	1.62±0.01 [b]	1.17±0.04 [a]	2.46±0.02 [b]	0.283±0.015 [a]	0.667±0.25 [a]
SF	2.46±0.15 [c]	1.55±0.06 [a]	1.55±0.06 [c]	0.731±0.006 [b]	0.433±0.15 [a]
BC	1.90±0.04 [ac]	2.79±0.15 [b]	3.37±0.12 [d]	0.708±0.003 [ab]	0.513±0.16 [a]

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte; NO – nitric oxide scavenging assay; LP – lipid peroxidation; FRAP – ferric reducing antioxidant power;
Mean ± SD, expressed as [1]IC_{50} values (mg/mL of extract) and [2]ascorbic acid equivalents (mg/mL of extract);
Mean values with unlike letters in columns show statistically significant differences ($p < 0.01$, Tukey's test);

All extracts showed DPPH• scavenger activity with IC_{50} values ranging from 1.62 mg/mL (MPR) to 2.46 mg/mL (SF). The IC_{50} values previous documented on literature for *S. ramosissima* ranged between 3.55 – 5.69 mg/mL (methanolic and ethanoic

extracts), which was higher than the IC_{50} obtained in this present thesis, therefore, these results showed higher radical scavenging activity than previous reported (Table 5). The ability of extracts to neutralize nitric oxide radical increased in the following order: BC>SF>MPR>MP. The highest activity regarding the neutralization of nitric oxide was accomplished by MP sample having a IC_{50} value of 1.07 mg/mL, however MPR and BC present similar IC_{50} ($p<0.01$). Regarding the IC_{50} values for other halophytes, *A. macrostachyum* showed a IC_{50} value of 0.60 ± 0.09 mg/mL, *Sarcocornia perennis alpine*, *Sarcocornia perennis* and *S. ramosissima* showed that the IC_{50} values higher than 10 mg/mL (5). Regarding these results, *S. ramosissima* extracts showed higher radical scavenging activity than previous reported (IC_{50}>10 mg/mL). The four extracts showed ABTS scavenger activity with IC_{50} values ranging from 0.283 mg/mL (MPR) to 0.731 mg/mL (SF). MP and MPR samples showed similar activity ($p<0.01$). The previous results documented for other halophytes ranged between 1.1 – 5.2 mg/mL, being the obtained radical scavenging activity for the four extracts higher than for halophytes from Southern Portugal, *A. macrostachyum*, *P. coronopus*, *M. edule* and *J. acutus* (119). After all, one can conclude that the *S. ramosissima* extracts from Marinha dos Peixinhos location (MP and MPR) showed higher capacity in the neutralization of DPPH, ABTS and NO radicals.

With regard to Fe^{2+}/ascorbate-induced lipid peroxidation, all extracts exhibited antioxidant activity, with IC_{50} values ranging from 1.55 to 5.55 mg/mL (SF and MP, respectively). Regarding these results, SF extract showed lower IC_{50} value ($p<0.01$) and BC extract showed higher IC_{50} value, since the IC_{50} value for MP extract was not statically different between the other extracts ($p<0.01$). As far as it is possible to know, these IC_{50} results were reported for the first time for halophytes species. The reducing power of extract (determined as mg/mL of extract) was higher in MP (0.706), followed by MPR (0.667), BC (0.523), and lower in SF (0.433). However, these results were not statically significant between the four species ($p<0.01$). The method is not well documented for *Salicornia* species and as far as it is possible to know, there are not results to compare between the genus. However, other halophytes have been studied, namely *Arthrocnemum macrostachyum*, *Sarcocornia perennis alpini* and *Sarcocornia perennis perennis* (5). All the results reported for these halophytes are expressed in IC_{50} values (0.84 - 6.55 mg/mL) or expressed in μmol Fe^{2+}/ 100 g (5,120).

The IC_{50} values shows that the MPR and MP are the extracts with higher antioxidant activity, followed SF and BC. Furthermore, all the samples showed that the antioxidant activity results are in accordance with previous results documented when it was possible to compare.

In order to reveal if there is any correlation between the antioxidant tests and the phenolic content, a Pearson correlation analysis was performed. The correlation factors between total phenolic content and antioxidants tests, expressed as the reciprocal value of the calculated IC_{50}, except for the FRAP assay for which determined acid ascorbic equivalents (AAE) values were used, are presented in Table 15.

Table 15- Pearson correlation coefficient between the amount of bioactive compounds and their antioxidant activities.

	DPPH	**TPC**	**FRAP**	**ABTS**	**NO**	**LP**
DPPH	1					
TPC	0.80	1				
FRAP	0.92	0.84	1			
ABTS	0.58	0.87	0.65	1		
NO	0.94	0.77	0.96	0.55	1	
LP	0.47	0.25	0.60	0.19	0.64	1

TPC – total phenolic content; LP – lipid peroxidation; NO – nitric oxide scavenger Capacity; FRAP – ferric reducing antioxidant activity;

The strongest correlation was found between total phenolic and examined activities, namely DPPH (r^2=0.80), ABTS (r^2=0.87), NO (r^2=0.77) and FRAP (r^2=0.84) assays. The obtained results confirm that the phenolic compounds are the main responsible for the reducing capacity of the extracts. Furthermore, high correlation was shown between FRAP and DPPH assays (r^2=0.92) as well as between FRAP and NO assays (r^2=0.96). On the other hand, the lack of correlation (Table 15) between total phenolic content in lipid peroxidation assay can be explained, since the thiobarbituric acid (TBA) test used for the detection of malondialdehyde (MDA) is nonspecific for this compound, and therefore other structurally similar substances present in plant extracts can react positively with TBA. In conclusion, considering the preliminary results for antioxidant tests and the screening of the phenolic profile for *S. ramosissima* extracts, this analysis suggests that the extracts present important biologically active phenolic and flavonoids compounds with scavenging and antioxidant activities. To improve the

knowledge about this halophyte, the values of IC_{50} can be express in mg of plant dw. The results are shown in Table 16.

Table 16- Necessary amount of plant (mg dwt) to inhibit 50% of DPPH, nitric oxide, lipid peroxidation and ABTS.

	Content (mg dw plant)			
	MP	**MPR**	**BC**	**SF**
DPPH	42.8	38.6	53.7	118.9
NO	24.7	27.9	43.8	134.9
LP	127.1	37.7	96.6	77.3
ABTS	7.12	6.74	20.0	35.35

MP - Marinha dos peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco River; SF – Santiago da Fonte;
NO – nitric oxide scavenger capacity; LP – lipid peroxidation;

2.2. Anti-inflammatory activity determination

2.2.1 Cytotoxicity results

For anti-inflammatory activity, the cytotoxicity of *S. ramosissima* extracts were investigated in human cell lines in their maximum concentration (300 mg/mL). The more common criteria used to monitor cell viability is cell counting. The trypan blue dye exclusion was performed, this vital stain (trypan blue) enters only compromised membranes of dead cells (121). The results are shown in Table 17.

Table 17 - Cytotoxicity results for extracts of *S. ramosissima*.

Extracts (300 mg/mL)	**% viable in test/ viable in control**
S. ramosissima	
MP	97.5 ± 2.81
MPR	96.1 ± 2.26
SF	100.7 ± 3.90
BC	99.4 ± 2.67

MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco; SF – Santiago da Fonte;
Values expressed as mean ± SD;

All the samples showed a percentage of viable cells higher than 95%. These results mean that the extracts were not toxic to the cells.

2.2.2. Inhibition of Prostaglandin E_2 and Thromboxane A_2 production

The anti-inflammatory activity of *S. ramosissima* extracts was studied by evaluating the effects on the production of arachidonic acid metabolites (PGE_2 and

TXA_2). The Figure 26 and the Figure 27 shows the results of different *S. ramosissima* extracts and aspirin in the production of two metabolites, TXA_2 and PGE_2, respectively.

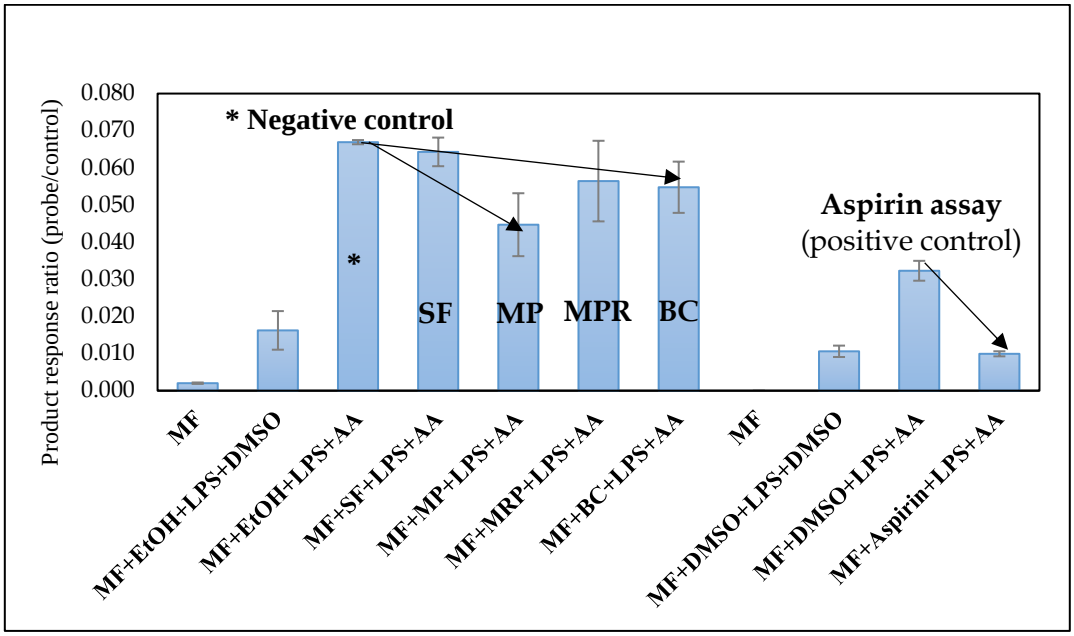

Figure 26- Effects of *S. ramosissima* extracts and aspirin in the production of TXA_2; MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco River; SF – Santiago da Fonte; MF – macrophages; LPS – lipopolysaccharide; AA – arachidonic acid; EtOH – ethanol; DMSO - dimethyl sulfoxide;

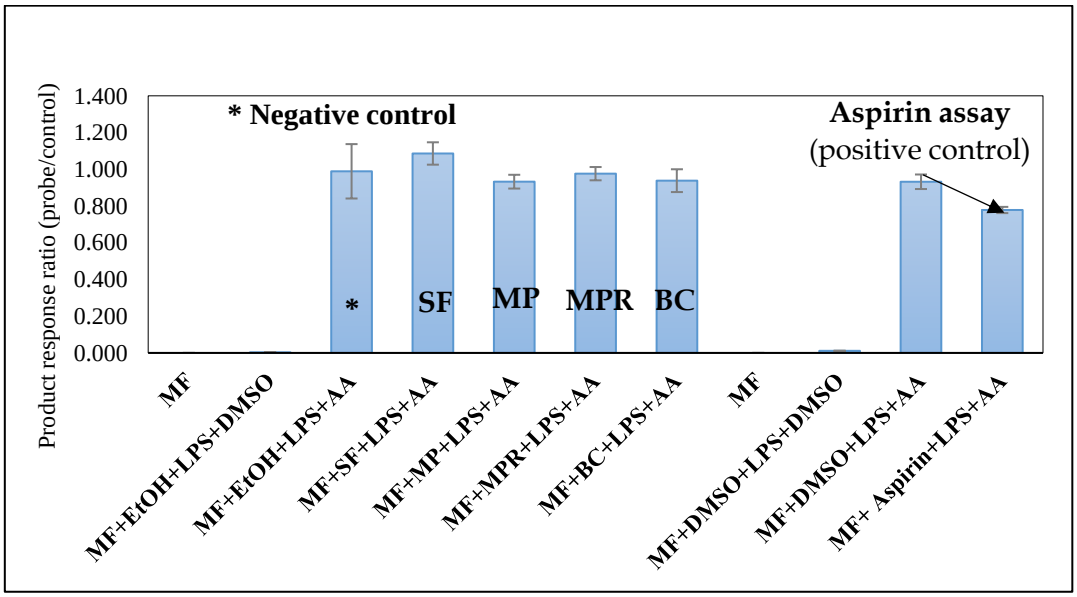

Figure 27 - Effects of *S. ramosissima* extracts and aspirin in the production of PGE_2; MP – Marinha dos Peixinhos; MPR – Marinha dos Peixinhos Red; BC – Boco River; SF – Santiago da Fonte; MF – macrophages; LPS – lipopolysaccharide; AA – arachidonic acid; EtOH – ethanol; DMSO – dimethyl sulfoxide;

Aspirin is a non-steroidal anti-inflammatory drug used in medicine to reduce the synthons of inflammation, fever and pain. This drug is non-selective and for that reason it inhibits the two forms of COX (COX-1 and COX-2) (122). MP and BC extracts showed as well as aspirin, inhibition of TXA_2 production. The other two extracts also presented a percentage of inhibition for this metabolite. However, these inhibitions were not significant when compared with the negative control. Aspirin showed a percentage of TXA_2 inhibition of 69.3% using the same methodology as for the extracts. MP and BC extracts showed a percentage of 33.2% and 18.1%, respectively. The mechanism of COX enzymes inhibition by natural products requires more studies. It is reported that some flavonoids can suppress the COX (COX-1 and COX-2) activity, namely naringenin, naringin, hesperetin, hesperidin, quercetin, luteolin, kaempferol, baicalein and myricetin and amentoflavone (123,124). Regarding the results obtained of the phenolic profile by LC-QqQ-MS, baicalein, quercetin, kaempferol, among other flavonoids were detected in the extracts and might be related to this reported activity. Furthermore, MP extract was the one with more flavonoids compounds detected (12, including amentoflavone, quercetin and kaempferol). This might explain the inhibition on TXA_2 production (33%). However more studies are required in order to evaluate this potential activity with different range of extracts concentrations, as well as in deep characterization of phenolic compounds (each sample in triplicate) and studies on specific enzymes activity to understand the inhibition by MP and BC extracts. BC extract present less phenolic compounds detected and total phenolic content than MP and MPR extracts. However, the composition of phenolic compounds in the extracts was only estimated and there were evaluated only specific standards. The BC extract might have other compounds that affect this inhibition on TXA_2 production.

For PGE_2, only aspirin showed anti-inflammatory activity, inhibiting the production of this metabolite. MP, MPR and BC extracts showed percentage of inhibition, however, these inhibitions were not significant when compared with the positive control. Aspirin inhibits the PGE_2 production in 18.0 %, less than for TXA_2 production. In this regard, *S. ramosissima* extracts might present potential anti-inflammatory activity, inhibiting the TXA_2 production, an eicosanoid related to platelets aggregation. The potential anti-inflammatory activity reported might be related with phenolic compounds detected among other compounds present in the extract.

Chapter IV – Conclusions and future work

1.1. Concluding remarks

The exploitation of *S. ramosissima* plant has gained increasing attention in the last few years. Important bioactive compounds, are reported on this plant, namely, sterols, phenolic compounds and unsaturated fatty acids. This present thesis accomplished the final objective, the characterization of phenolic and lipophilic compounds as well as the evaluation of antioxidant and anti-inflammatory activity of *S. ramosissima*. For this analysis, the fructification stage of this plant was studied, collected in three locations of Ria de Aveiro lagoon. Regarding the *S. ramosissima* metabolites profiling, this master thesis focused on two main extracts:

I. Lipophilic fraction (dichloromethane extractives)
II. Polar fraction (methanol extractives)

Total phenolic content was performed, the results ranged between 1391-3398 mg/100g dw. These values were similar to previous reported (Table 3), MP and MPR samples showed higher phenolic content (2734-3398 mg/100g dry weight). A detailed chemical analysis of *S. ramosissima* plant revealed the presence of 31 phenolic compounds, 22 were reported for the first time in this matrix. From these non-reported phenolic compounds for *S. ramosissima* species, three were reported for the first time in *Salicornia* genus. The phenolic content of 15 compounds was estimated, expressed in μg/g of extract. The total content ranged between 5.6-1676.6 μg/g of extract. The screening of phenolic compounds was different between the four samples. The MP and MPR extracts were found to have higher amount of phenolic compounds (1012.9 and 1676.6 μg/g, respectively). These results allow verification of the MP and MPR extracts form Marinha dos Peixinhos location as the most promising to extract phenolic compounds. The total phenolic content accessed by LC-QqQ-MS was lower than for the analysis from Folin-Ciocalteu method. However, the quantification in LC-MS was only estimated and the identification was performed for a limit number of standards.

Regarding the lipophilic fraction, 35 compounds were detected, being the unsaturated fatty acids the main family present, followed by sterols. Linoleic and linolenic acids, β-sitosterol and stigmasterol were the main components detected. The total content ranged between 22.5 – 67.6 mg/g of extract. The total content between the four samples was different. MPR and SF extracts were the richest in lipophilic compounds (46.3 and 67.6 mg/g of extract, respectively). These results allowed to identify higher levels for

unsaturated fatty acids and sterols in samples than previous reported for this matrix (Table 2).

The minerals content was also evaluated, the quantification of essential minerals (Na, K, Mg and Se) and toxic compounds was performed. Sodium (Na) is the major mineral present in this halophyte. The results showed a higher content for this mineral than previous reported for this species (Table 4). Sodium levels ranged between 95-123 mg/g dry weight. For potassium (K), the results were also higher than previous reported on this species (Table 4), ranging between 10-18 mg/g dry weight. On the other hand, magnesium content was in accordance with previous results (Table 3), ranging between 6.3-11 mg/g dry weight. To our best knowledge, selenium was quantified for the first time in this matrix and the levels ranged between 1.2-1.4 μg/g dry weight.

In order to evaluate the potential of *S. ramosissima* plant as a source of bioactive compounds, the Table 18 shows an overview of the composition of this plant in fresh weight.

Table 18 – Lipophilic, phenolic and mineral estimated content in *S. ramosissima* fresh plant.

	Totals	**Composition (mg/g fresh plant)**
Lipophilic content	Saturated fatty acids	0.2-2.7
	Unsaturated fatty acids	0.2-6.5
	Sterols	0.1-0.5
Phenolic content (Gallic acid equivalents)		2.6-6.1
Mineral content	Sodium	17.9-21.2
	Potassium	1.8-3.2
	Magnesium	1.2-1.9
	Selenium	0.00021-0.00029
Total		42.0 (4.2%)

Furthermore, considering 5g of fresh edible plant to be used in a salad, it would correspond to 6.0-7.1% of sodium recommended daily intake, 1.9-2.6% of selenium recommended daily intake, 1.3-2.1% of magnesium recommended daily intake and 0.2-0.3% of potassium recommended daily intake. These results allowed to identify this plant as a potential salt substitute, showing lower sodium levels per gram and with presence of other essential minerals.

The total antioxidant activity was evaluated through different methods in order to extensively characterize the antioxidant potential of *S. ramosissima* extracts. IC_{50} values for DPPH and NO were higher than the previous detected in this species and other halophytes (Table 5). FRAP assay and lipid peroxidation were reported for the first time in this species as well as in *Salicornia* genus. MPR and MP extracts from Marinha dos Peixinhos presented higher antioxidant and radical scavenging activity (IC_{50} $DPPH\cdot$ = 1.62-1.87 mg/mL; IC_{50} $ABTS^{+}\cdot$ =0.283-0.308 mg/mL; IC_{50} NO=1.07-1.17 mg/mL; ferric reducing antioxidant activity = 0.667-0.706 mg/mL of extract in acid ascorbic equivalents). These results are in accordance with the phenolic composition for these two extracts. They present higher phenolic compounds detected as well as total phenolic content (2734 and 3398 mg GAE/100g dry weight). Regarding anti-inflammatory activity of the *S. ramosissima* extracts. The production of PGE_2 was not affected by the extracts, however, MP and BC extracts affected the TXA_2 production, inhibiting the metabolite production in 33.2% and 18.1%, respectively. The inhibition might be related with phenolic compounds detected among other compounds present in the extracts. Aspirin inhibits TXA_2 (69.3%) and PGE_2 (18.0%) production, using the same methodology. These results allowed to conclude that *S. ramosissima* extracts might present potential anti-inflammatory activity, inhibiting TXA_2 production.

In order to evaluate the toxic components for safety use as a food product, lead and mercury were analysed, the levels were below the limit of quantification. Therefore, these values could not be convert as fresh weight, being impossible to compare with legislated values (0.5µg – 1.0 for Hg and 0.2 µg/g for Pb).

The acquired knowledge reveals that this halophyte can be exploited as a source of bioactive compounds (unsaturated fatty acids, sterols and phenolic compounds) as well as salt substitute, presenting lower sodium levels per gram when compared to salt. Since, higher sodium intakes are related to cardiovascular diseases and hypertension, this plant can be used as dietary adjuvant in this civilization diseases. The bioactive compounds detected might be related with antioxidant and anti-inflammatory activities exhibited by this halophyte.

1.2. Future work

Following the main objective of this thesis and considering the results and conclusions obtained, there are some challenges that could be considered:

- ✓ Study the polar fraction by LC-MS (each sample in triplicate) in order to quantify the identified compounds;
- ✓ Testing different concentration ranges on arachidonic acid metabolism to infer if at different dosage, the extracts inhibit PGE_2 production;
- ✓ Study the biological activity of *S. ramosissima* lipophilic extracts.
- ✓ Valorization of *S. ramosissima*, taking advantage of the chemical composition and the biological effects associated, developing new food formulations with potential health benefits (e.g. lower sodium intake);

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Appendix

Appendix 1

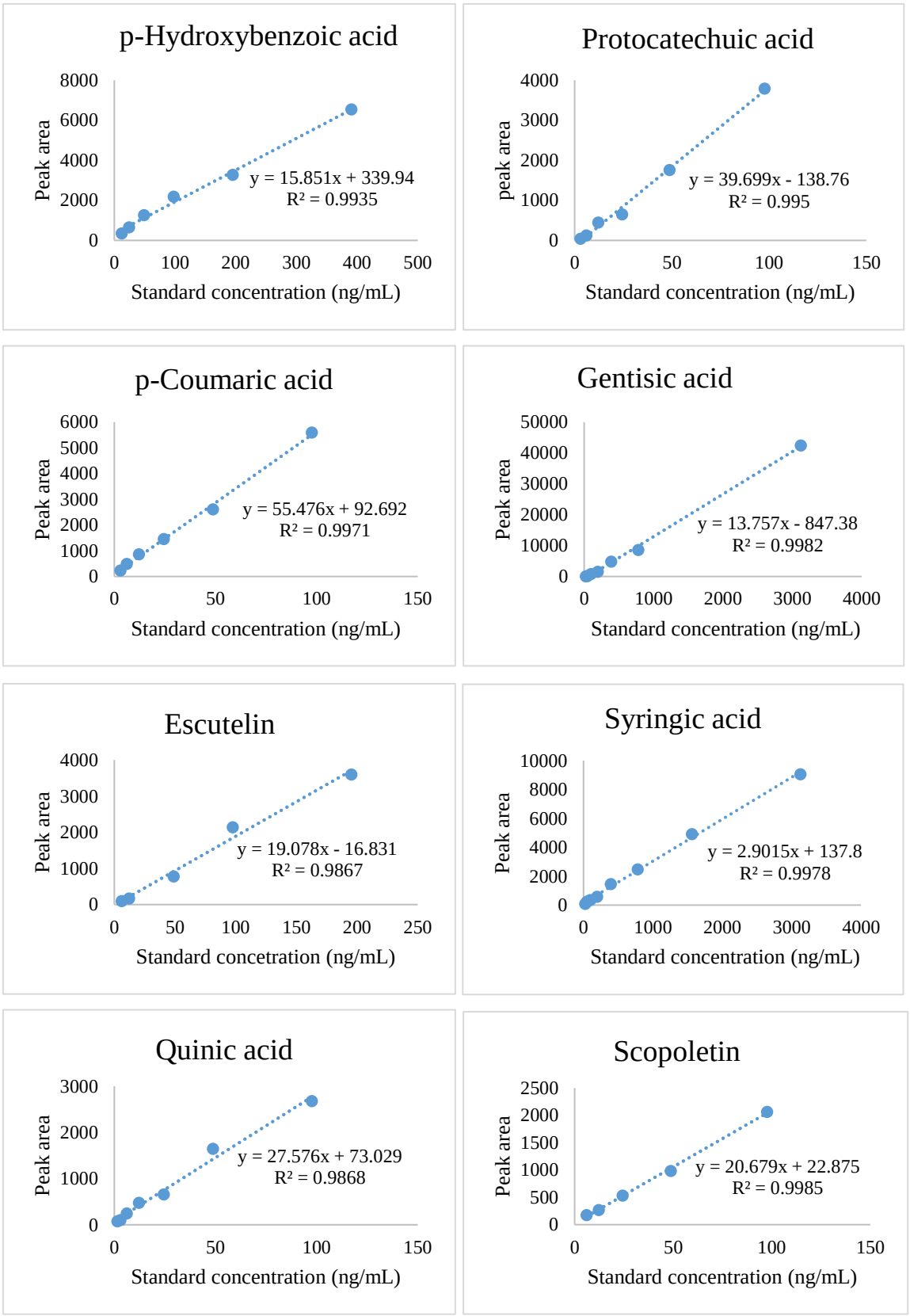


Figure 28- Calibration curves for determinate the concentrations of standard compounds in the extracts.

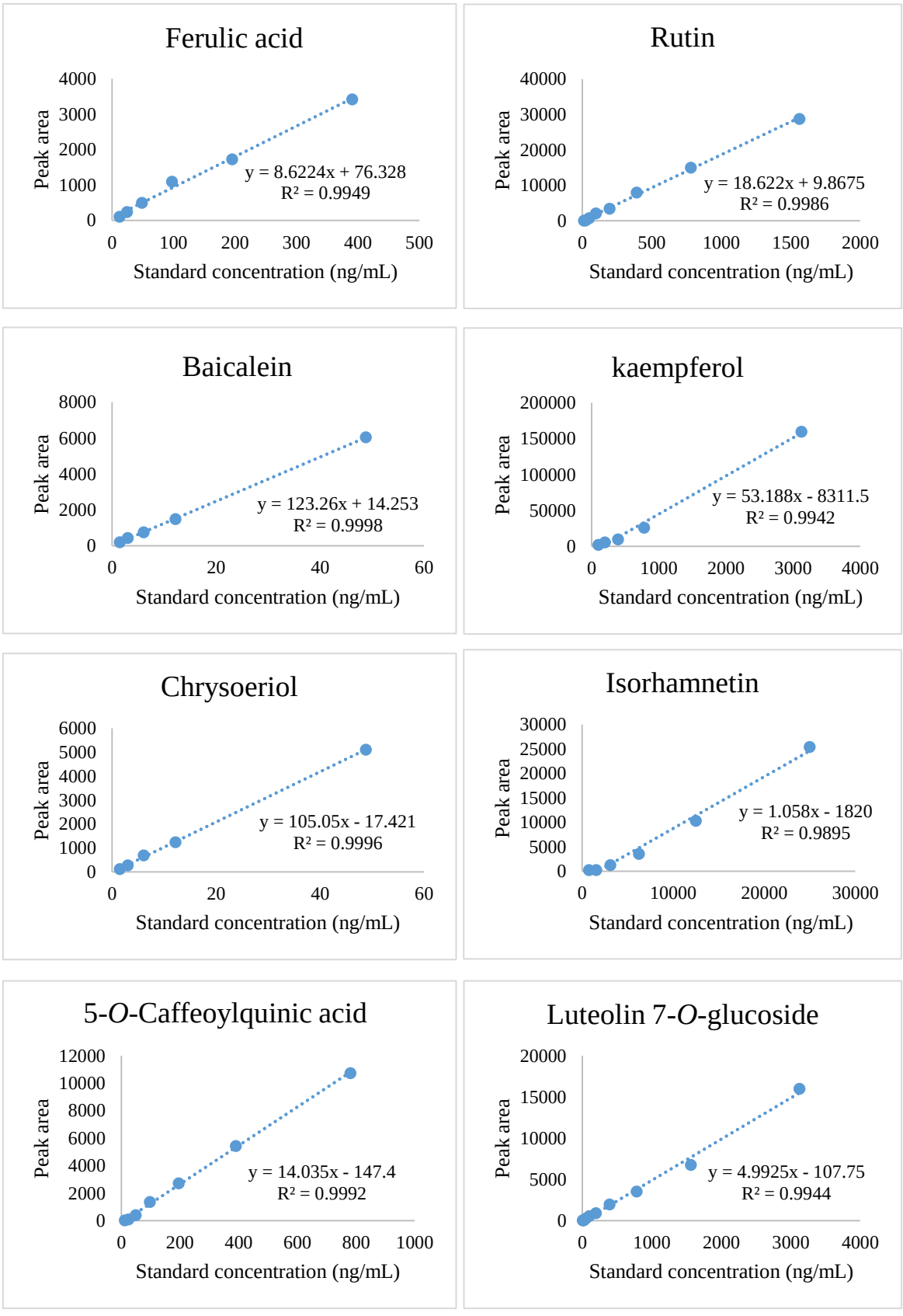


Figure 28- (continued).

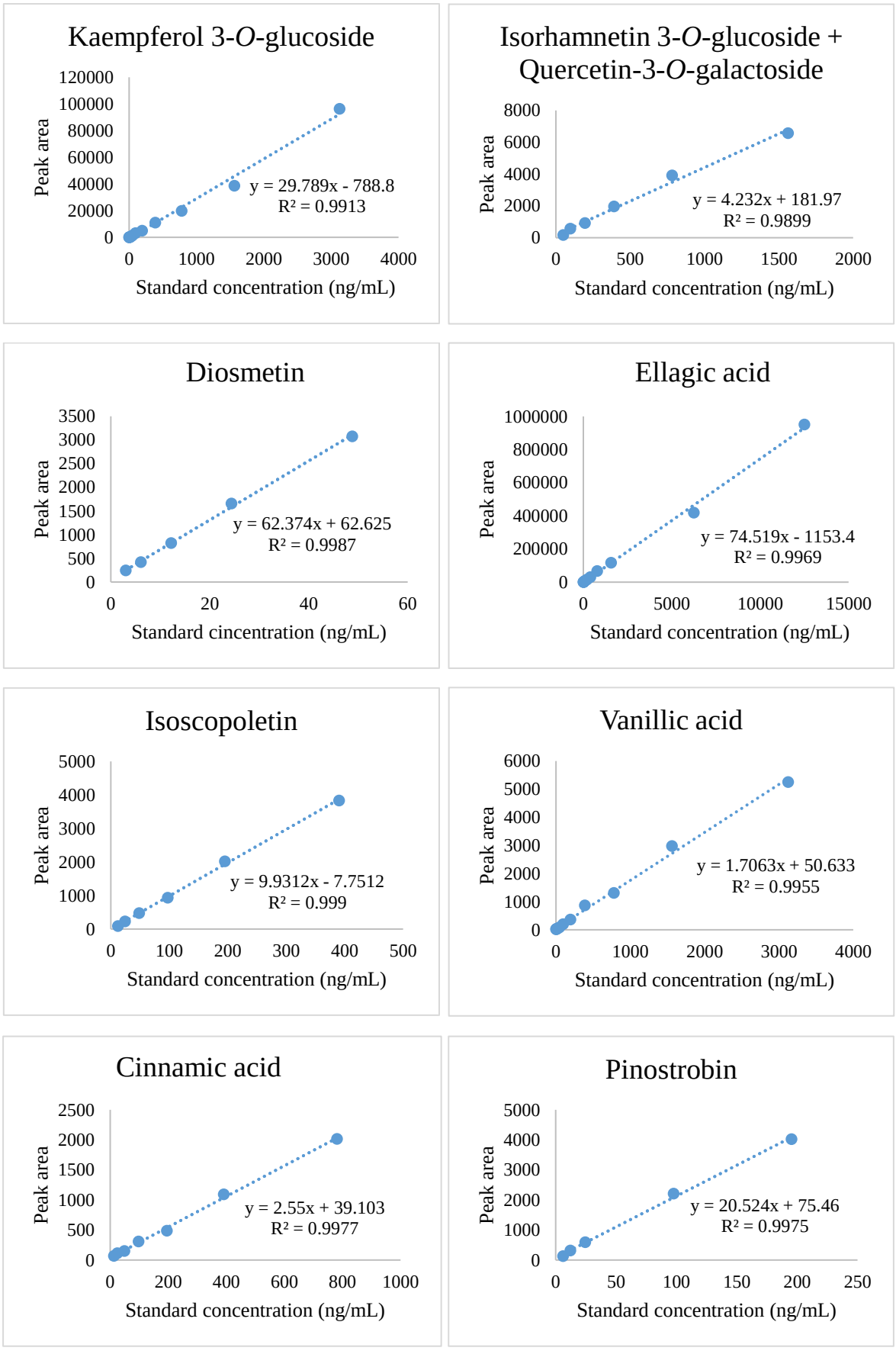
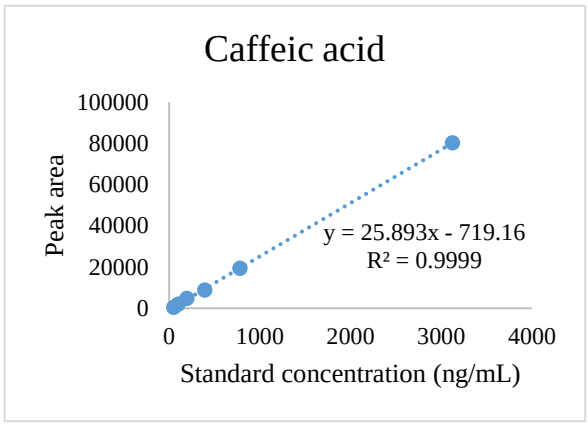
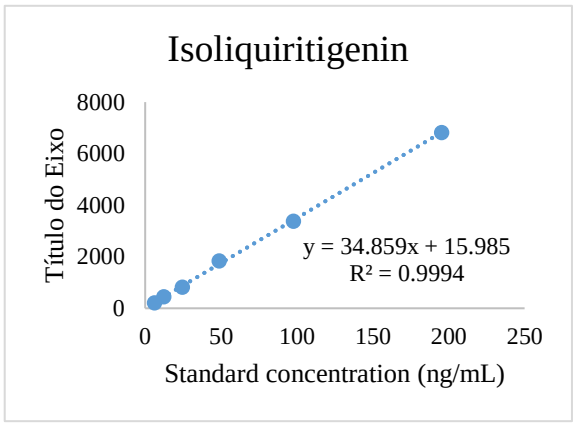
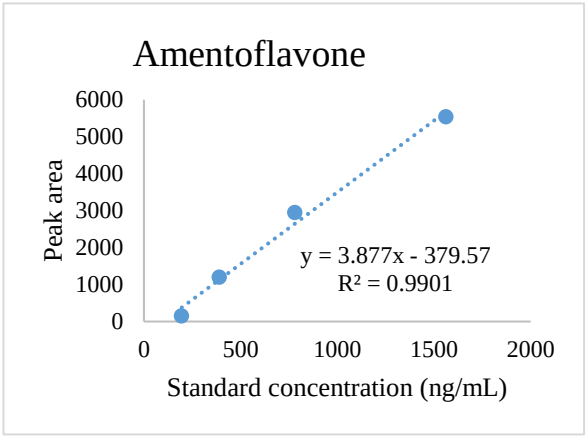
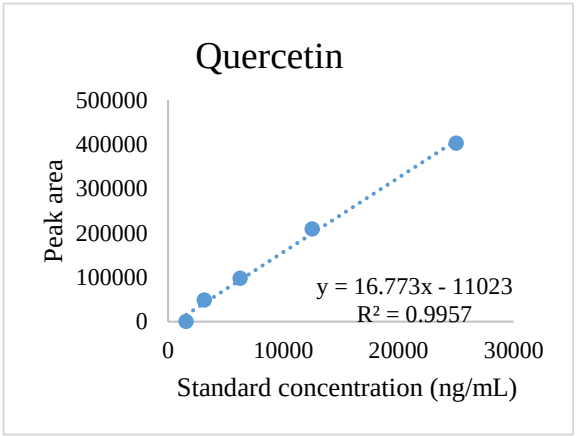


Figure 28- (Continued).









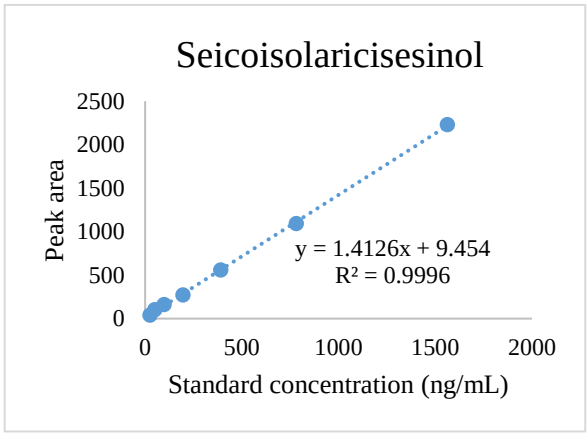


Figure 28- (Continued).

Table 19 - Calibration data used for the quantification of phenolic compounds in *S. ramosissima* polar extract.

Compound	Calibration curve[1]	r^2	LOD[2]	LOQ[2]
			ng/mL	
p-Hydroxybenzoic acid	y = 15.851x + 339.94	0.9935	39.2	130.7
Cinnamic acid	y = 2.5500x + 39,103	0.9995	44.0	146.7
Protocatechuic acid	y = 39.699x - 138.76	0.9953	8.60	28.7
Gentisic acid	y = 13.757x - 847.38	0.9982	154.7	515.7
p-Coumaric acid	y = 55.476x + 92.692	0.9971	6.50	21.7
Vanillic acid	y = 1.7063x + 50.633	0.9955	206.4	688.1
Escutelin	y = 19.078x - 16.831	0.9867	31.4	104.2
Caffeic acid	y = 25.893x - 719.16	0.9999	45.8	152.8
Quinic acid	y = 27.576x + 73.029	0.9951	9.84	32.8
Scopoletin	y = 20.679x + 22.875	0.9985	4.96	16.5
Ferulic acid	y = 8.6224x + 76.328	0.9949	34.8	116.0
Syringic acid	y = 2.9015x + 137.8	0.9978	164.6	548.6
Baicalein	y = 123.26x + 14.253	0.9998	0.85	2.83
Kaempferol	y = 53.188x - 8311.5	0.9942	332.7	1109.0
Chrysoeriol	y = 105.05x - 17.421	0.9996	1.39	4.64
Quercetin	y = 16.773x - 11023	0.9957	2160.9	7203.9
Isorhamnetin	y = 1.058x - 1820	0.9895	3203.0	10676.8
5-*O*-Caffeoylquinic acid	y = 14.035x - 147.4	0.9992	26.5	88.4
Secoisolaricisesinol	y = 1.4126x + 9.454	0.9996	36.9	122.9
Luteolin 7-*O*-glucoside	y = 4.9925x - 107.75	0.9944	239.5	798.4
Kaempferol 3-*O*-glucoside	y = 29.789x - 788.8	0.9913	277.1	923.6
Isorhamnetin 3-*O*-glucoside + Quercetin 3-*O*-galactoside	y = 4.232x + 181.97	0.9899	196.4	654.5
Amentoflavone	y = 3.877x – 379.57	0.9901	658.9	2196.4
Rutin	y = 18.622x + 9.8675	0.9986	65.9	219.7
Isoscopoletin	y = 9.9312x - 7.7512	0.9994	15.3	50.9
Isoliquiritigenin	y = 34.859x + 15.985	0.9994	5.75	19.17
Pinostrobin	y = 20.524x + 75.46	0.9975	13.8	46.1
Diosmetin	y = 62.374x + 62.625	0.9987	2.32	7.75
Ellagic acid	y = 74.519x - 1153.4	0.9969	696.6	2321.9

[b] y: peak area; x: concentration in ng/mL; [c] LOD: limit of detention; LOQ: limit of quantification;

Appendix 2

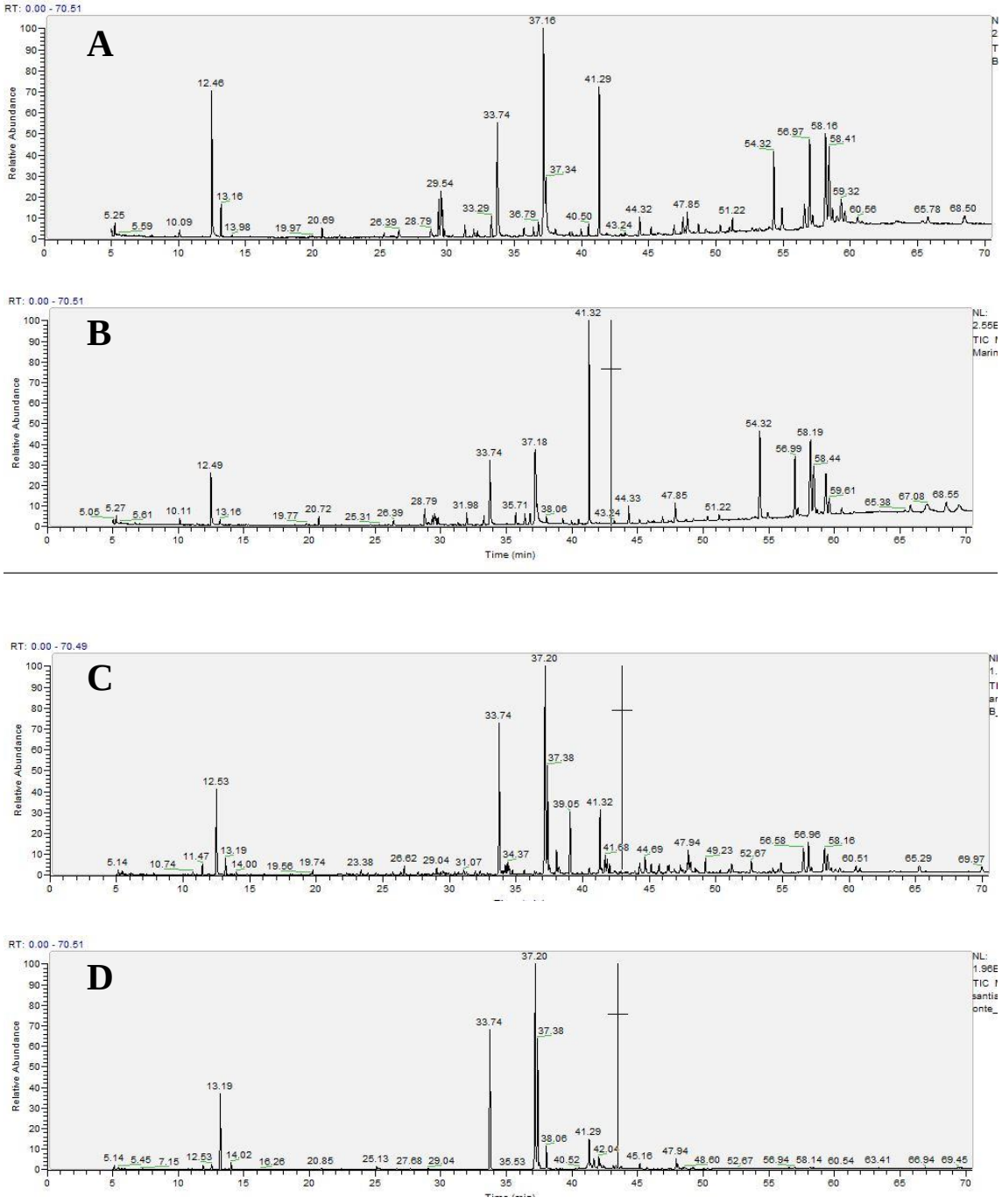


Figure 29 - GC-MS chromatogram of the TMS-derivatized dichloromethane extracts of *S. ramosissima* plant harvested in Ria de Aveiro, Portugal. A- Boco river; B – Marinha dos Peixinhos; C – Marinha dos Peixinhos (MPR sample); D – Santiago da Fonte.

Appendix 3

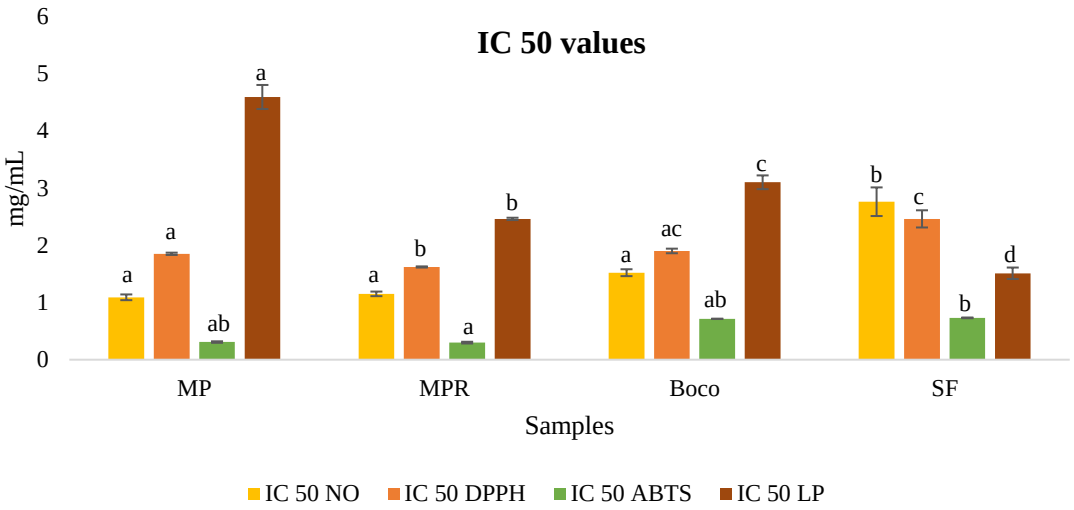


Figure 30- Representation of the IC 50 values for each sample. Values expressed as mean ± SD; Mean values with unlike letters in IC 50 test show statistically significant differences ($p < 0.01$, Tukey's test).