NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Biology

IN VITRO BIOACESSIBILITY AND BIOACTIVITY OF PHENOLIC COMPOUNDS FROM HALOPHYTE PLANTS

MASTER IN BIOTECHNOLOGY

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ABSTRACT

Halophyte plants have a high content of minerals (*e.g.* sodium, potassium and manganese) and phytochemicals with antioxidant properties (*e.g*. phenolic compounds). For this reason, there is a growing interest in the use of these plants as food. However, the bioactivity of these plants is dependent on the bioaccessibility of their bioactive compounds, namely phenolic compounds.

The main objective of this thesis is to evaluate the impact of the *in vitro* gastrointestinal digestion process on the phenolic composition and bioactivity of halophyte plants. To achieve this objective, two halophyte plants were selected and submitted to an *in vitro* digestion process, in order to assess the bioaccessibility and antioxidant activity of phenolic compounds throughout the phases of the digestive process.

In the first part, the phenolic composition of seven halophyte plants were determined by HPLC-DAD-ESI-MS/MS, and further quantified by HPLC-DAD and the colorimetric method - Folin-Ciocalteu method. Two plants *(Salicornia ramosissima* and *Sarcocornia fruticosa*) were selected for the in vitro digestion studies due to their high content and variety of phenolic compounds.

The analysis of digestive fractions revealed that there was an increase in total phenolic compounds from oral to gastric phase and a consequent decrease in intestinal phase. The % of bioaccessible phenolics of *S. ramosissima* were 6.8%,18.4% and 7.4% and for *S. fruticosa* were 32.3%, 67.5% and 51.5%, for oral, gastric and intestinal fractions, respectively. The same trend was observed in the antioxidant activity assays (ORAC and HOSC values). HPLC-DAD analysis demonstrated that gallocatechin, caffeoylquinic acids and caffeoylquinic acid derivatives were identified as the major phenolics found in both plants. Most of these compounds proved to be poorly bioaccessible as they were not detected in the intestinal fraction.

***Key words*:** Halophyte plants, *in vitro* digestion, *Salicornia ramosissima* and *Sarcocornia fruticosa*

RESUMO

As plantas halófitas apresentam alto teor de minerais (*e. g.* sódio, potássio e manganês) e fitoquímicos com propriedades antioxidantes (*e. g.* compostos fenólicos). Por esse motivo, há um interesse crescente na utilização dessas plantas como alimento. No entanto, a bioatividade destas plantas está dependente da bioacessibilidade dos seus compostos bioativos, nomeadamente compostos fenólicos.

O principal objetivo desta dissertação é avaliar o impacto do processo de digestão gastrointestinal *in vitro* na composição fenólica e bioatividade de plantas halófitas. Para atingir este objetivo, duas plantas halófitas foram selecionadas e submetidas a um processo de digestão *in vitro,* a fim de avaliar a bioacessibilidade e atividade antioxidante dos compostos fenólicos ao longo das fases do processo digestivo.

Na primeira parte, a composição fenólica de sete plantas halófitas foi caracterizada por HPLC-DAD-ESI-MS/MS, e quantificada por HPLC-DAD e pelo método colorimétrico - método de Folin-Ciocalteu. Duas plantas (*Salicornia ramosissima e Sarcocornia fruticosa*) foram selecionadas para os estudos de digestão *in vitro* devido ao seu alto teor e variedade de compostos fenólicos .

A análise das frações digestivas revelou que houve aumento dos compostos fenólicos totais da fase oral para a gástrica e consequente diminuição na fase intestinal. As % de fenólicos bioacessíveis presentes na *S. ramosissima* foram 6.8 %,18.4% e 7.4% e para *S. fruticosa* foram 32.3%, 67.5% e 51.5%, respetivamente para as fases oral, gástrica e intestinal, para a digestão de ambas as plantas. A mesma tendência foi observada nos ensaios de atividade antioxidante (ORAC e HOSC). A análise de HPLC-DAD demonstrou que galocatequina, ácidos cafeoilquínicos e derivados de ácidos cafeoilquínicos foram identificados como sendo os principais fenólicos encontrados em ambas as plantas. A maioria destes compostos mostrou-se pouco bioacessível, não sendo detetado na fração intestinal.

Palavras-chave: plantas halófitas, digestão *in vitro, Salicornia ramosissima e Sarcocornia fruticos*

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ACRONYMS

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
DAD	Diode-Array Detection
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
ESDAC	European Soil Data Center
ESI	Electrospray ionization
FDA	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh weight
GAE	Gallic acid equivalents
HCV	Hepatitis C Virus
HOSC	Hydroxyl Radical Scavenging Capacity
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
m/z	Mass to charge ratio
MS/MS	Tandem Mass Spectrometry
ORAC	Oxygen Radical Antioxidant Capacity
ROS	Reactive Oxygen Species
RP	Reverse Phase
R_T	Retention time
SFCA	Surfactant-free Cellulose Acetate

SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid
TAME	*p*-Toluene-sulfonyl-L-arginine methyl ester
TCA	Trichloroacetic acid
TEAC	Trolox Equivalent Antioxidant Capacity
TP	Total Phenolics
TPC	Total Phenolic Content
Trolox	6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid

SYMBOLS

°C	Celsius
µg	Microgram
µM	Micromolar
Ca^{2+}	Calcium
$CaCO_3$	Calcium carbonate
$FeCl_3$	Iron(III) chloride
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
IC_{50}	Half maximal inhibitory concentration
kg	Kilogram
Mg	Magnesium
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
Na^+	Sodium
Na_2SO_4	Sodium sulfate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NO	Nitric oxide radical

OH	Hydroxyl group
s	Second
r	Pearson’s r correlation

1

INTRODUCTION

1.1 Halophyte plants

Salinity is among the main abiotic stressors that most impair plant development and agricultural output in large terrestrial areas of the planet [1], [2]. Currently, salinity affects circa 800 million hectares throughout the world, with Australia, the Middle East, Africa and Latin America being the most impacted by this abiotic stress [3]. According to European Soil Data Center (ESDAC), the European regions most affected by salinity are the Iberian Peninsula, Ukraine, and the Carpathian and Caspian hydrographic basins [4]. In the European community space, Southern European countries are the most affected with almost one million hectares damaged in some form by salinization [4]. In relation to Portugal, saline soils are mostly found on the western and southern coasts of Portugal, more specifically on the banks of rivers and estuaries, although they may also be found in some agricultural areas in the interior of the Alentejo [5]. Due to many causes such as inadequate agricultural practices, irrigation with salt water, very high surface evaporation, weathering of native rocks, and reducing precipitation, the number of places impacted by salinization on a global scale is growing at a rate of 10% each year [6].

The challenge of rising soil salinity, along with the development of the human population in this century, has led to a quest for novel solutions not only for enhancing the productivity of existing crops but also for new crops that can thrive in degraded habitats (such as those affected by salinity) and consume little resources (such as water and fertilizers) [7]. Halophytes plants have high physiological plasticity and can drive in an environment where this abiotic stress (salinity) is present [8]. Because of this capacity, Panta *et al.* (2014) claim that halophyte plants may be utilized as a cost-effective and environmentally sustainable alternative to traditional crops, that rely on high-quality water and soils- [9].

Halophytes are plants capable of growing and thriving in high concentrations of salt, concentrations at which 99% of other plants do not survive. Flowers *et al.* (2008) give us a definition of halophyte plants as those that can " *complete the life cycle in a salt concentration of at least 200 mm NaCl under conditions similar to those that might be encountered in the natural environment"* [11]. These plants can grow in a diverse number of saline environments including salt marshes, coastal dunes, oases, inland saline depressions, steppes, and anthropogenic salines [11], [12].

Halophytes have several evolutionary adaptations that may explain the salinity tolerance of these plants and the ability to overcome the stresses placed in their tissues. Currently, very little is known about these evolutionary strategies, but among the strategies already known are specialized salt glands or compartmentalization of salts in the vacuole. All evolutionary adaptation strategies to high salinity attempt to:

- Maintain cellular turgor and transpiration [13];
- Restore the homeostatic conditions necessary for the absorption and distribution of nutrients [13];
- Mitigate the functional and structural damage done to cells [13];

In certain halophytes species, the high concentration of salts is not a threat to them but it is needed for its existence. For example, the sodium ion, that accumulates in the vacuole of cells, has been shown to play an important role in the growth and development of *Salicornia europaea*, stimulating cell expansion and shoot succulence [1]. Other halophytes such as *Bassia indica* and *Limonium bicolor* have been proved to have a positive halotropism, a directional and sodium-specific root movement to acquire the correct salt concentration required for normal physiological development [14].

There are several classifications proposed for the halophytes plants, which are based on characteristics such as their mechanisms of adaptation, salt needs, or/and biotope traits [15]. There is a geographical classification that divides halophytes two groups: Xerohalophytes, which are well acclimated to low-humid conditions and deserts, and Hydrohalophytes, which are found in brackish wetlands [12]. Other classifications distinguish between obligatory, facultative, and habitat-indifferent halophytes based on ecophysiological properties [16], [17]. Obligate halophytes require an abundant amount of salt for their normal development; facultative halophytes are plants that can withstand high salinities but are also able to grow where this stress is not present [16], [17]. However, they prefer to grow in saline habitats due to the high competition for resources in non-saline habitats; and, lastly, habitat-indifferent halophytes are plants that can tolerate saline circumstances but prefer to exist in areas without this stress[16], [17]. Grigore *et al.* (2010) established a new type of halophyte categorization that considers ecological value, anatomical adaptations, and their importance, as well as general halophyte strategies [18]. They classified halophytes as extreme halophytes, which are found only (or almost exclusively) in saline environments, and mesohalophytes, which can grow in a variety of habitats, not just those affected by salinity, though they exhibit relevant physiological characteristics when this stress is present [18].

These salt-tolerant crops are becoming a viable alternative to traditional crops as soil salinity rises and water quality declines [10], [19]. The notion of "biosaline agriculture" arose as a result of this environmental problem, which is defined as "*Profitable and improved agricultural practices using saline land and saline irrigation water with the purpose to achieve better production through the sustainable and integrated use of genetic resources (plants, animals, fish, insects, and microorganisms) avoiding expensive soil recovery measures* [19]." Growing market demand, and the good nutritional profile of halophytes make the promotion of *"biosaline agriculture"* a necessity [9], [20]. Other options have been implemented, such as growing plants in soilless and controlled environments using hydroponics and aquaponics techniques. Some companies in Portugal, such as RiaFresh©, already produce and

commercialize plants that are grown entirely in hydroponics [21]. Others, such as Pinheiro *et al.* (2016), are working on aquaponics systems that utilize effluents from the production of Pacific white shrimp to simultaneously produce *Salicornia* [22]. Changes in the environmental circumstances in which plants develop, on the other hand, cause changes in plant metabolism, which affect the synthesis and accumulation of primary and secondary metabolites [23]. As a result, hydroponic and/or aquaponic plants are likely to differ chemically from those found in nature. Maciel *et al.* (2016) corroborated this by demonstrating that halophyte plants grown in aquaponics (using effluent from fish production and imitating the abiotic conditions of the plant's environment) had a greater concentration of glycolipids with ω-3 fatty acids than plants grown in the wild [24].

Aside from the possibility of acquiring value-added products, halophyte plants can also play an ecologically important function by decontaminating and rehabilitating degraded soils [25]. Yunusa *et al.* (2003) recommend the use of halophyte plants as "primer plants", plants whose primary function is to improve soil conditions so that subsequent plants can thrive properly [26]. They point when plants were used in bioremediation, there was a longer-lasting improvement in soil physical constraints than when chemical techniques were used [26]. Rabhi *et al.* (2008) investigated the application of three halophyte species in the bioremediation of soils impacted by salinization in arid areas: *Sesuvium portulacastrum, Suaeda fruticosa* and *Arthrocnemum indicum* [27]. By absorbing large amounts of sodium from the soil, all the plants they employed significantly reduced the electrical conductivity and salinity of the soils [27]. Between the three plants, the authors verified that *Sesuvium portulacastrum* plants had the greatest ability to take salt from the soil, removing the equivalent of 26% of the initial amount of sodium present in the soil [27]. Rabhi *et al.* (2010) proved that the cultivation of *Sesuvium portulacastrum* in soil affected by salinization was efficient in reducing the salinity, having a bioremediation capacity equivalent to 1 ton of sodium removed per hectare [28]. Nasir *et al.* (2009) also tested the application of three halophytes plants (*Atreplix hallimus , Atriplex numularia* and *Tamarix aphylla*) to alter the chemical composition of soil impacted by salinization, finding that these plants are able to remove between 3.45-4.38 kg of sodium chloride per square meter [29].

1.2 Halophytes plants and their food applications

The commercial interest in the halophyte plants have been rising due to their potential non-agricultural and agricultural applications [20]. Halophyte plants can be cultivated or harvested to be used for food, as a source of bioactive compounds with medicinal and nutraceutical applications, to obtain other value-added products, among others [20].

Halophytes have a nutritional profile suitable for human consumption due to their content in several salts and micro and macronutrients [20], [30]. These plants contain high concentrations of ω-3 polysaturated fatty acids, like palmitic acid, and are also abundant in minerals, such as sodium and manganese [30]. Halophytes can also be applied as medicinal plants as they synthesize and accumulate a large and diverse amount of secondary compounds (such as saponins, alkaloids, carotenoids, proanthocyanidins, tannins, phenolic compounds) with a vast number of pharmacological and biological

activities [31]. These antioxidant compounds are essential for the human diet and play an important role from the point of view of human health. For example, the intake of carotenoids has a protective role in various disorders mediated by ROS, such as neurological and cardiovascular diseases, various types of cancer, eye diseases, among others [32].

Some halophyte plants have a perceived salinity and other organoleptic attributes (such as appearance, aroma, texture, flavor, and aftertaste) that make them a suitable alternative to conventional salt - a 'biosalt' [33], [34]. This 'biosalt 'obtained from halophyte plants is a vegetable-derived salt that has a low sodium content (variations between 8.36-17.4 mg/g of DW in S*alicornia* compared with 38.5-38.9 g/100 g (385-389 mg/g) of table salt[1]) combined with other minerals (e.g. magnesium, calcium, potassium, manganese) and it is enriched in bioactive substances such as phenolic compounds and other nutrients [21], [33], [35]. Its lower sodium content, when compared to the table salt, makes its food application useful to prevent the onset of hypertension and other cardiovascular-related diseases [33]. In Europe, some species of halophytes such as *Salicornia europaea*, *Salicornia bigelovii*, *Salicornia ramossisima* are being sold as vegetable and salad leaves at rather high prices and are essentially used in gourmet cuisine [36], [37].

The application of halophyte plants in several food products has been proposed by several authors:

i) Dried spice - Renna *et al.* (2013) created a dried spice, that can also be applied as a food colorant, using *Chritmum maritimum* [38];
ii) Functional beverage - Pereira *et al.* (2017) proposed developing a functional beverage from decoctions and infusions of *Chritmum maritimum* [39];
iii) Cracker - Clavel-Coibrié *et al.* (2021) formulated a cracker containing 5% *Sarcocornia perennis* with high acceptance from a sensory panel [40];
iv) Salt alternative- Shin and Lee (2013) created microgranules comprising powder combined 10% with aqueous extract of *Salicornia herbacea* covered with a coater [41]. Kim *et al.* (2014), proposed the utilization of 1 % *Salicornia herbacea* powder in sausage manufacture to lower salt content [42]. The use of *Salicornia ramosissima* was proposed by Lopes *et al.* (2017) as a way to minimize salt in bread [43];

Cardoso *et al.* (2022) and Custódio *et a*l. (2021) studied the receptivity of *Salicornia* sp. as a vegetable, sold as fresh leaves but also as green salt. Although unknown to most panelists, the general impression was favorable, with a considerable number of people (mainly women and people looking to diversify their diet) having a high intention to buy these products [33], [44].

1.3 Phenolic compounds from halophytes plants

Phenolic compounds are metabolites that have at least one hydroxyl groups that are linked directly to one or more benzene aromatic ring [45] Phenolics compounds are very common secondary metabolites that are generally found in conjugated form (such as glycosides or esters) or in association

[1] Data from ' Food and Safety Authority of Ireland'

with other phenolics compounds [45], [46]. Tannins, phenolic acids, and flavonoids are the three most significant types of dietary phenolics [46], [47]. In nature, flavonoids are found as glycosides with one or more sugar connected by a carbon–carbon bound or an OH group , whereas phenolic acids are generally found in conjugated or insoluble forms [48].

Abiotic stressors on halophytes plants included drought, severe temperatures, diverse salinity levels, inundation, and lack of oxygen circumstances [49]. Halophytes have a series of adaptation mechanisms to cope with an imbalance in ROS production caused by excessive salinity and other abiotic stressors [49]. The production and accumulation of numerous defensive chemicals, including phenolics, are among the adaptive mechanism produced by halophyte plants to deal with these stressors [49]. The production and accumulation of flavonoids in a halophyte, *Prosopis strombulifera*, grown in soil containing Na_2SO_4 was confirmed by Reginato *et al.* (2014), indicating that these chemicals may play a role in preventing oxidative damage caused by high levels of salt stress [50].

A study of 45 medicinal plants made an interesting discovery by determining that phenolic compounds among the other secondary compounds, are the compounds that most contribute to the antioxidant activities of these plants [51] In fact, phenolic compounds are best known for their potent antioxidant action, but they've also been related to anti-aging, anti-diabetic, anti-cancer, neuroprotective, antiviral, anti-inflammatory, and other health-promoting properties [8] However, it's vital to remember that in order for phenolic compounds to exercise their antioxidant action, they must be adequately digested, absorbed, and be able to reach the target organ [52].

1.4 *Salicornia sp.*

Figure 1.1: ***Salicornia ramosissima*** (image from Riafresh® website: https://www.riafresh.com/index.php/pt/produtos/item/4-salicornia)

Salicornia sp, is a plant genus that belongs to the *Amaranthaceae* family [53]. There are several common names by which these plants are known, such as samphire, crow's foot greens, sea asparagus, sea beans, glasswort, pickleweed [54] About 25-30 different species belong to this genus with the

following plants being the most commonly studied: *Salicornia persica*, *Salicornia herbacea*, *Salicornia ramosissima*, *Salicornia maritima*, *Salicornia virginica*, *Salicornia brachiata*, *Salicornia bigelovii* and *Salicornia europaea*, **Figure 1.1** [44], [54].

The only species of the genus *Salicornia* that is native in Portugal is *Salicornia ramosissima* (*S. ramosissima*), which develops preferentially on the coast, in the middle and upper marsh, and salt marshes. It is distributed over the western part of Europe and the western part of the region of the Mediterranean. It can be found all over the coast of the Iberian Peninsula, except in Minho region [55].

S. ramosissima is an annual plant characterized by not producing leaves, and for having interesting organoleptic characteristics such as a pleasant texture and a salty and juicy flavor [21], [44]. Because of these organoleptic properties, the plant has a strong economic potential. In fact, *S. ramosissima* is already available in gourmet stores as a mixed salad with green leaves [21].

Concerning nutritional profile, *Salicornia persica* cultivated under seawater irrigation, exhibited a content of protein 2.53 mg/ g FW and a total shoot lipid content of up to 2.41 mg/ g FW, with Ω-3 fatty acids corresponding to 47.6 % of the total fatty acids [37]. Furthermore, compounds with antioxidants properties such as phenolic compounds (with 121 mg GAE/100 g FW), and carotenoids (with 4690 µg/100 g FW) are present [37], [56]. *S. ramosissima* from 'Ria de Aveiro, Portugal' has a low protein (1.6% of FW), total dietary fiber (1.0% of FW), and carbohydrate content (2.9% of FW), but a high fatty acid content, such as linolenic (33.5 % of total fatty acid), linoleic (24.1% of total fatty acid), and palmitic (24.0% of total fatty acid) acids [57]. Cardoso *et al.(*2021), also studied the nutritional profile of *S. ramosissima* from 'Ria de Aveiro' and found a nutritional profile similar to that described by Alves *et al.* (2021), with protein corresponding to 2%, fiber corresponding to 3.3% and carbohydrate corresponding to 2.6 % of FW [44].

1.4.1 Phenolic compounds of *Salicornia sp*.

S. ramosissima, in addition to being considered a source of essential minerals, is also a source of bioactive compounds such as phenolic acids and flavonoids[44] The value of phenolic compounds in this plant has already been reported by several authors, ranging from 7.41 mg GAE/ g DW to 27.44 mg GAE/ g DW, depending on the species, production method, extraction method, and processing method [57]–[60].

There are recent studies where some phenolic compounds have already been identified and quantified in species belonging to the genus *Salicornia*, namely:

i) Surget *et al.* (2015) analyze the phenolic compounds present in an ethyl acetate fraction of *S. ramosissima*, and identified ten different phenolic compounds for the first time in this species: 7 phenolic acids (dicaffeoylquinic acid, caffeoyl-hydrocaffeoylquinic acid, dihydrocaffeoyl quinic acid, caffeoylquinic acid, hydrocaffeoylquinic acid, caffeic acid, hydrocaffeic acid) and 3 flavonoids (isorhamnetin, isorhamnetin glucoside and quercetin glucoside) [61];
ii) Panth *et al.* (2016) analyzed *Salicornia europaea* extract and registered the existence of three different compounds: trans-ferulic acid; p-coumaric acid and 5-

(hydroxymethyl)furfural. These compounds were also quantified, having the trans-ferulic acid, the p-coumaric acid and the 5-(hydroxymethyl)furfural, 2.60 ± 0.33, 3.19 ± 0.47 and 18.20 ± 1.80, µg/g, respectively [62];

iii) Chung *et al.* (2005) isolated from *Salicornia herbacea* a new chlorogenic acid derivative compound using a bioassay-directed chromatographic separation technique, which It was called tungtungmadic acid (also known as 3-caffeoyl, 4-dihydrocaffeoyl quinic acid) [63];

iv) Lee *et al.* (2004) isolated for the first time in *Salicornia herbacea* methanolic extract, a phenolic compound, named isorhamnetin-3-O-β-D-glucopyranoside [64];

v) Oliveira- Alves *et al.* (2021) analyzed the phenolic composition of *S. ramosissima* subject to different drying processes. They found that caffeoylquinic acid derivatives and quercetin glycosides are the main compounds identified [57];

vi) Pinto *et al.* (2021) in their study of the applicability of residues *from S. ramosissima*, found that caffeoylquinic acid derivatives are the main compounds identified. Of these, three compounds stand out for having a high concentration: 4-caffeoylquinic acid, 1,4-dicapheoylquinic acid and 3,5-dicapheoylquinic acid had concentrations of 5.54, 6.78 and 8.24 mg/g dw, respectively [59];

vii) Silva *et al.* (2021), in their comparative research of two extractions method of *S. ramosissima*, found that gallic acid and myricetin were the components with the greatest concentration [60];

1.4.2 Bioactivities of *Salicornia sp.*

Several biological activities with some benefit to human health have been attributed to *Salicornia* sp. extracts [61]. Zengin *et al.* (2008) studied the capacity of *Salicornia europeae* to inhibit the activity of two brain enzymes, namely butyrylcholinesterase and acetylcholinesterase. The authors proved a moderate neuroprotective effect of *S. europeae*, with the ethyl acetate extract and the methanolic extract showing 1.99 mg of galantine equivalents (GALAE)/ g of extract and 2.34 mg of GALAE/g of extract for the anti-cholinergic activity, respectively. The plant also proved to have a butyrylcholinesterase inhibitory capacity of 2.19 mg of GALAE/ g of extract for ethyl acetate extract and a value of 1.88 of GALAE/g of extract for methanolic extract [65]. Pinto *et al.* (2021) studied the neuroprotective effect of an aqueous extract of residues of *S. ramosissima.* This extract prove to have an anti-cholinergic activity, that was found to be concentration-dependent, with values ranging from 23.84% (with the lowest concentration tested: 250 µg/mL) to 32.34% (with the highest concentration tested: 1000 µg/mL) [66].

Ferreira *et al.* (2016) studied the protective effect of ethanolic extract of *S. ramossisima* on testicular toxicity induced by carbon tetrachloride. The application of the ethanolic extract of *S. ramosissima*, before the application of the agent that cause the damage - the carbon tetrachloride, prevents the appearance of lesions at histopathological level [67].

Hwang *et al.* (2007) studied the antidiabetic effect of an ethanolic extract of *Salicornia herbacea* (*S. herbacea*) in a mice model of type 2 diabetes. Mice were fed with a diet supplemented with 1 % desalted ethanolic extract of *S. herbacea*. The authors verified that the mice fed with this diet presented a hypolipidemic effect with a decrease in plasma triglyceride and total cholesterol level. They also concluded that the extract of *S, herbacea* had an inhibitory effect against the pancreatic lipase which could be the cause of this hypolipidemic effect [68].

Silva *et al.* (2021) evaluated the *in vitro* antioxidant and antiradical activities of aqueous extracts of *S. ramosissima* obtained in two different extraction methods: a conventional extraction and a microwave-assisted extraction [69]. The microwave assisted-extraction displayed higher antioxidant and antiradical activities (65.56 µmol FSE/g dw for the Ferric Reducing Antioxidant Power (FRAP) Assay and 1.74 µg AAE/g dw for the ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Radical Scavenging Activity Assay) than the conventional extraction for both assays performed (60.61 µmol FSE/g dw for the FRAP assay and 15.55 µg AAE/g dw for ABTS assay) [69]. Barreira *et al.* (2017) also evaluate the antioxidant capacity of *S. ramosissima*, reporting that ethanolic extracts of *Salicornia ramosissima* could scavenge DPPH radical with values of 5.69 ± 0.09 mg/mL [30]. Antunes *et al.* (2021) studied the antioxidant activity of *S. ramossisima* collected in May and July and stored for 14 days at 4°C. In this study was verified that the plant's harvest in May had a significantly higher antioxidant activity than the ones harvest in July in both antioxidant assays (Trolox Equivalent Antioxidant Capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC)). For the plants harvest in May, the TEAC had a value of 1.90 ± 0.13 mM Trolox/100 g and the ORAC had a value of 15.97 ± 0.23 mM Trolox/100 g, For the plant harvest in July, the TEAC had a value of 0.37 ± 0.01 mM Trolox/100 g and the ORAC had a value of 2.74 ± 0.05 Trolox/100 g [70].

Elatif *et al.* (2020) evaluated the cytotoxicity activity of the methanolic extract of *Salicornia fruticosa* (*S. fruticosa*) against four human cancer cells lines: MCF-7, A549, HepG2, and HCT-116. They proved that the *S.fruticosa* extract had a dose-depending suppressor effect in the growth of the tested cells, having a more effective effect in HCT-116 [71].

Oliveira-Alves *et al.* (2021) examined the antihypertensive and antiproliferative effects of *S. ramosissima* dried by two drying processes (freeze-drying and oven-drying). They obtained IC_{50} values of 18.96 mg/mL for the freeze-dried and 24.56 mg/mL for the oven-drying in the angiotensin-converting activity assay. While the antiproliferative assay using a colon cancer cell model (HT29) showed EC_{50} values of 17.24 mg/mL for the freeze-drying and 17.56 mg/mL for the oven-drying) [57].

Kang *et al.* (2011) tested the cytotoxic and antioxidant activities of diverse fractions of *Salicornia herbacea*'s seed extracts. From all the fractions, the ethyl acetate fraction showed the strongest cytotoxicity effect against HT-29 and HTC 116 cell lines, having values of IC_{50} of 50.4 µg/mL for HT-29 cells and 2.34 µg/mL for HCT 116 cells. In relation to the antioxidant activity, in all the assays realized (NO, ABTS and DPPH) the ethyl acetate fraction presented the highest values (IC_{50} values of 0.2 , 0.87 and 0.18 mg/mL for NO, ABTS and DPPH essays, respectively) [72].

1.5 *Sarcorcornia sp.*

Figure 1.2: ***Sarcocornia fruticosa*** (Image from Riafresh® website:https://www.riafresh.com/index.php/pt/produtos/item/9-sarcocornia)

The genus *Sarcorcornia* , like other *Salicornioideae*, belongs to the *Amaranthaceae* family and includes species with succulent, articulate, and photosynthetic stems [73], [74]. Six species belonging to the genus *Sarcocornia* A.J. Scott had been identified in the western Mediterranean: *Sarcocornia carinata, Sarcoconia hispanicain, Sarcocornia alpini, Sarcocornia pruinosa, Sarcocornia perennis.* These plants are native to the salt marshes and estuarine of the Mediterranean and Atlantic coasts but also to the inland saltmarshes of Portugal and Spain [75], **Figure 1.2.**

Sarcocornia fruticosa (*S. fruticosa*) is commonly found in the tidal zones generally growing in zones with the highest soil salinities, and less frequently in temporarily flooded zones [76], [77]. Species that belong to *Salicornia* and *Sarcocornia* genus are close to each other, because of that they are often mistaken and can only be distinguished by their life form, being *Sarcocornia sp.* always perennial and *Salicornia sp.* annual [78].

Regarding the nutritional content, the lipid content of *Sarcocornia fruticosa* is up to 2.06 mg/g FW, with the ω3 fatty acid representing 41.2% of the content. It has a protein content of 253 mg/100 g FW, a total phenolic content of 121 mg GAE/100 g FW and β-carotene content of 4690 µg/100 g FW [37], [56]. *Sarcocornia fruticosa* showed a content of protein between 1.0 and 1.5% of FW, a total dietary fiber between 0.7 and 3.5% of FW and fat content between 0.5 and 0.7% of FW, with the values depending on local of cultivation and the type of cultivation [79].

1.5.1 Phenolic content of *Sarcocornia sp.*

Bertin *et al*. (2013) in their research of the nutritional profile of two distinct populations *of Sarcocornia Ambigua,* identified 15 phenolic compounds: isoquercetin, kaempferol, narigin, quercetin,

galatin, syringaldehyde, scopoletin, chlorogenic acid, sinapic acid , syringic acid, caffeic acid, ferulic acid, vanilic acid, cinnamic and p-coumaric acid. The most abundant compounds identified in both populations were ferulic acid, caffeic acid, vanillic acid , p-coumaric acid, kaempferol, and galangin [80].

Castañeda-Loaiza *et al.* (2020) compared the methanolic extracts of *Sarcocornia fructicosa* collected from wild populations present in the saltmarshes of Spain and Portugal with hydroponic cultivated plants. The authors verified that the cultivated *Sarcocornia fructicosa* showed a highest phenolic compounds content than the plants collected either in Spain or Portugal, being the 3,4 - dihydroxybenzoic acid, catechin hydrate and chlorogenic acid the main phenolic acids quantified in this plant [81].

Hawas *et al.* (2018) isolated six flavonoids glycosides from 70% aqueous methanol extract of dry *S, fruticosa* leaves collected on Saudi coast. The authors identified 5 known flavonols: isorhamnetin, isorhamnetin 3-O-(2″,6″-O-α-dirhamnosyl)-β-galactoside, rhamnazin 3-O-rutinoside, isorhamnetin 3-O-(6″- O-α-rhamnosyl)-β-galactoside. Furthermore, a novel flavonol triglycoside, designated as rhamnazin 3-O-2G-rhamnorutinoside, was identified by these authors for the first time in this plant [82].

Costa *et al.* (2018) studied the bioactive compounds of three biotypes (red, green, and pink) of sea asparagus *Sarcocornia ambigua*. Concerning the phenolic compounds, the main compounds identified in *S. ambigua* tissues were gallic acid and kaempferol, followed by quercetin and hydroxybenzoic acid. The red biotype's reproductive portions contained the greatest concentrations of these compounds [83].

Sánchez-Gavilán *et al.* (2021) studied the bioactive compounds of different species of two wild halophytes genus, *Sarcocornia* and *Arthocnemum*, present in the coastal marshes of Guadiana and Tinto rivers, and certain parts of the interior of Iberian Peninsula [84]. Several populations of three species belonging to *Sarcocornia* genus was were analyzed: *Sarcocornia perennis, Sarcocornia pruinosa and Sarcocornia alpine* [84] Five phenolic acids were identified in these species: caffeic, p-coumaric, veratric, salicytic, trans-cinnamic acid, being the salicylic acid and the transcinnamic acid the major compounds [84] In addition, some flavonoids were also identified, with all the three quercetin were also found in *S. pruinosa* [84].

1.6 Bioactivities of *Sarcocornia sp.*

Costa *et al.* (2018) studied the antioxidant activity of fresh vegetative and reproductive segments of three biotypes (green, red and pink biotypes) of sea asparagus *Sarcorcornia ambigua.* The extracts obtained from vegetative and reproductive segments of *Sarcorcornia ambigua* biotypes showed antioxidant properties in the ABTS assay, with a range of values of 3.4 - 4.9 mmol of TEAC/ 100 g of FW. The vegetative segments showed a significantly lower antioxidant activity than reproductive segments, with the pink and red reproductive segments having the more particularly pronounced antioxidant activity, with the 4.9 and 4.8 mmol of TEAC/ 100 g of FW respectively. The authors proved that the antioxidant activity of sea asparagus extracts and the total phenolic acids content had a positive correlation ($r = 0.670$) [83].

Barreira *et al.* (2017) studied the antioxidant activity of two subspecies of *Sarcocornia perennis* (*Sarcocornia perennis alpini* and *Sarcocornia perennis perennis*) harvested in Algarve, Portugal. The antioxidant activity of ethanolic extracts of *Sarcocornia perennis alpini* and *Sarcocornia perennis perennis*, by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, had IC_{50} values of 8.04 and 11.5 mg/mL, respectively. In the ferric reducing antioxidant power (FRAP) assay, the ethanolic extracts of *Sarcocornia perennis alpini* and *Sarcocornia perennis perennis* proved to have iron reducing capabilities with IC_{50} values of 6.55 and 4.57 mg/mL, respectively [30].

Gargouri *et al.* (2013) studied the *in vitro* cytoprotective property of *Sarcocornia perennis* extracts in renal cells (HEK293 kidney cells) after exposure to lead. Lead exposition caused several effects at a cellular level, such as a reduction in cell viability, loss of cohesion of the cells and cell distortion. This exposition also induced intense oxidative stress with the production of free radicals, such as superoxide anion, as well as lipid peroxidation. The damages indicated above were mitigated by supplementing *S. perennis* extract to the medium containing lead. The authors proved that *S.perennis* extract exerts a cytoprotective effect by reducing the ROS levels and the consequent oxidative stress [85].

Hawas *et al.* (2018) tested if the aqueous-alcoholic extract of *S. fruticosa* and its extracted flavonol glycosides were capable of inhibiting the Hepatitis C virus (HCV) protease [64], [68]. The crude extract displayed a good anti-HCV protease activity with an IC_{50} of 10.5 µg/mL. Regarding the flavonol glycosides, rhamnazin tri-glycoside had the strongest level of inhibition with IC_{50} value of 8.9 µM [82].

1.7 Bioaccessibility and bioavailability

Halophytes plants have a high nutritional value and a strong potentiality as a functional food, Although the plants can have a high nutritional value and/or a high content of bioactive compounds, this does not mean that all the plant content will be fully utilized by the human body. Some plants contain significant levels of bioactive compounds, but most of these compounds are not completely metabolized or absorbed in the intestine, during gastrointestinal digestion [87]. Therefore, this is the reason why the scientific concepts of bioaccessibility and bioavailability were developed [87].

The term bioavailability was developed by the FDA (Food and Drug Administration (FDA)), in order to determine which proportion of the main active compounds of a drug was absorbed and entered into the blood circulation, and then what will be available to exert its activity in its place of action [88]. This concept was later adapted to the field of nutrients and bioactive compounds. Silvia Cozzolino (2016) defined bioavailability as "*the proportion of nutrients that are available for normal metabolic and physiological processes* " [88].

The release from the food matrix, the chemical state of the nutrients, probable interactions with other component present in the matrix, and the production of persistent molecules that are slowly digested, can all affect the availability of the nutrient [89], [90]. In almost every situation, the chemical, enzymatic and physical digestion processes are affected by the matrix food's physical qualities [89], [90].

The bioavailability of phenolic compounds present in food is affected by diverse factors resulting from the digestive process, including the rate of digestion, first-pass effect, metabolic modification, fermentation carried out by microorganisms present in the colon [91]. Intrinsic characteristics of phenolic compounds such as their degree of polymerization and the presence of functional groups are determinants for the bioavailability of phenolic compounds. These characteristics affect the solubility and consequently the absorption and metabolization of phenolic compounds in their own pathway [91]. Furthermore, true bioavailability and bioaccessibility of phenolics are also affected by the processing method applied to the food and interaction with other compounds present in the food matrix [91].

Bioaccessibility, absorption, and transformation are the three key stages studied in nutraceutical bioavailability research. The first part consists of the bioaccessibility, which is defined as "*the portion of nutrient that is released by the food matrix into the gastrointestinal cavity and has the potential to be absorbed during digestion*" [92]. Absorption is "*the movement of nutraceuticals from the gastrointestinal fluids to the systemic circulation*". Only a fraction can pass through the epithelial layer of the intestine [93]. Finally, transformation consists of chemical and biochemical changes of compounds present in the gastrointestinal fluid (*e.g*. conjugation reactions with addiction of methyl, glucuronides and/or sulfate groups) [46]**,** [94]. The compounds are metabolized and converted into smaller molecules by the liver and intestine.

There are several *in vivo* and *in vitro* approaches that elucidated the bioavailability and the bioaccessibility of the nutrients. *In vivo* models (*e.g*. human and animals model) provides more specific and more accurate information about bioaccessibility and bioavailability, however, these studies are not conducted because they are not only lengthy, and expensive trials but also entail some ethical constraints [95], [96]. As an alternative, *in vitro* models *(e.g*. static model as developed by INFOGEST and use of Caco-2 cells) were developed. These models are simple, inexpensive, reproducible, and make it possible to test a large numbers of conditions and/or samples [95], [96].

1.7.1 *In vitro* digestion models

Many *in vitro* digestion models have been developed over the years to simulate the digestion process in order to avoid human testing as these are expensive, resource intensive and ethically debatable [97]. These models range from simple static models to complex and dynamic models [98], [99]. Several characteristics connected to the operating mode are what distinguishes each model, and these parameters include:

- The number and type of digestive stages [80], [81];
- The chemical formulation of the digestive fluids employed in each stage [80], [81];
- The mechanical loads and fluid flows that are imposed at each stage of the process [80], [81];

1.8 INFOGEST *in vitro* digestion model

Aiming at allowing a direct analysis and comparison of the results obtained by different research groups, the INFOGEST Cost action consortium proposed a standardized and practical static digestion approach that can be used for a variety of purposes and that can be altered to accommodate a specific requirement [97]. Based on important physiological data, the authors standardized the following parameters:

- The number of phases, which was defined as 3 (oral, gastric, intestinal) [97], [100];
- The concentration of electrolytes in oral (SSF), gastric (SGF) and intestinal (SIF) simulated fluids [97], [100];
- The pH and duration of digestive phases (2 min at pH 7 for the salivary; 2h at pH 3 for the gastric and 2 h at pH 7 for the intestinal) [97], [100];
- The inclusion of enzymes depending on their enzymatic activity and bile extracts according to their bile acid content [97], [100];

Prior experimental determination of the concentration of bile salts and activity of all digestive enzymes, as detailed by Minekus *et al.* (2014) in the supplementary information, is a critical step in ensuring the effectiveness of the *in vitro* digestion process, **step 1** in **Figure 1.3** [97], [101].

1.8.1 Oral phase

Unless the matrix contains starch, the oral phase is unnecessary for liquid food matrices due to the short residence duration of liquids in the mouth.[100], [101] For solid foods, an appropriate amount of food must be crushed in order to simulate chewing and the mixture with the SSF must have a tomato pulp consistency. [97], [100], [101] In the oral phase, food is diluted with simulated salivary fluid (SSF) in a 1:1 portion, in the presence or absence of salivary amylase. If used, food contact time with salivary amylase is restricted to 2 minutes at pH 7, **step 2 and step 3** in **Figure 1.3** [97], [101].

1.8.2 Gastric phase

In the gastric phase, the protein digestion of food will occur mediated by the presence of pepsin and HCl. In the gastric phase, the oral bolus is combined with simulated gastric fluid (SGF) and pepsin and incubated under agitation at pH 3.0 for 2 h [97], [101]. The digestion of lipids in the stomach is not simulated since there are no enzymes on the market that can simulate the action of human gastric lipase, **step 4** in **Figure 1.3**.

1.8.3 Intestinal phase

There are two ways to simulate enzyme activity in the intestinal phase. In the first option, a porcine-derived enzymatic extract of the pancreas (pancreatin), which includes all pancreatic enzymes in quantities comparable to those seen in humans is used [97], [100]. The amount of pancreatin to be added will be based on the enzyme activity determined by the trypsin or lipase assay, in case the food has a

high-fat content [97], [100]. Alternatively, individual enzymes (lipase, amylase, chymotrypsin and trypsin) can be used [97], [100]. In the intestinal phase, the solution from the gastric phase is mixed with simulated intestinal fluid (SIF), bile salts and pancreatic enzymes, (the individual enzymes or pancreatin) and incubated for 2 h at pH 7, **step 5** in **Figure 1.3**. [97], [101].

Sampling requires some treatments to ensure that enzymes are inactivated and do not operate after completion of the *in vitro* digestion procedure. Several options are suggested to achieve this purpose such as; the use of enzyme inhibitors such as pefabloc, rapid freezing of the sample with liquid nitrogen and/or gastric pH neutralization, **step 6** in **Figure 1.3** [97], [100], [102].

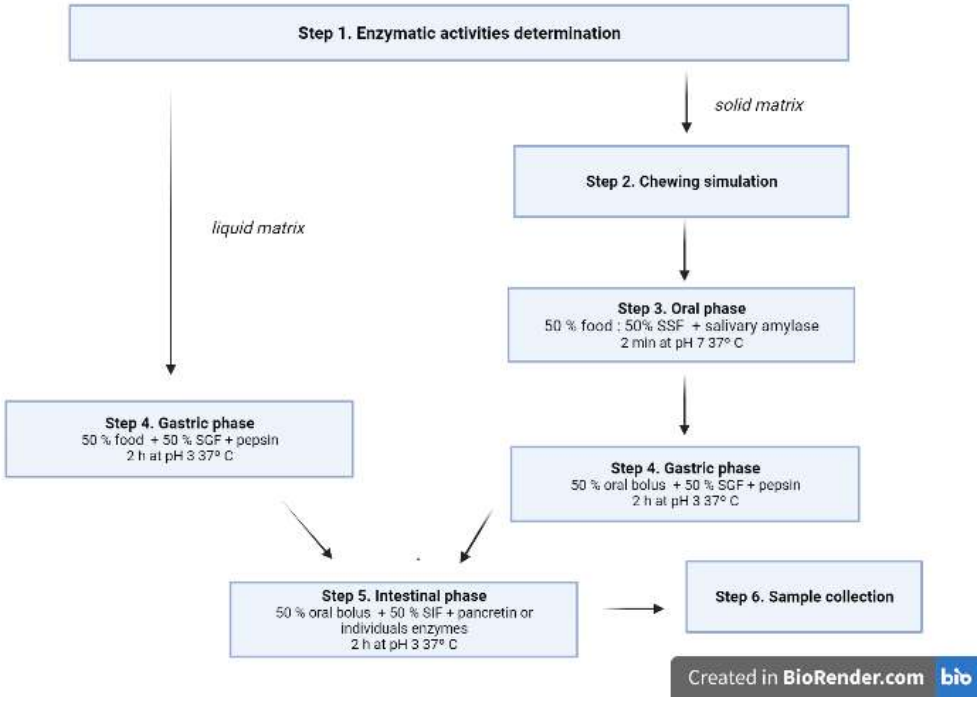

Figure 1.3: Schematic representation of INFOGEST *in vitro* model Adapted from Minekus *et al* .(2014) and Brodkorb *et al*. (2019)

1.8.4 Advantages and disadvantages of INFOGEST *in vitro* digestion model

The model developed by the consortium has several positive points such as:

1. Standardization of parameters and digestion conditions, thus allowing an effective comparison of the obtained results [97], [101];
2. The model accommodates some adjustments and suggestions, taking into account some intrinsic properties of the food and other parameters related with the reagents and enzymes (such as the type and animal origin of the selected enzyme) [97], [101];

But because it is a static model, it has some limitations, such as:

1. Use of enzymes of animal origin [97], [101], [103];
2. Inability to simulate the flow of food through the gastrointestinal compartments (gastric emptying rate and intestinal transit time) [97], [101], [103];

3. Impossibility of simulating the secretion of saliva [97], [101], [103];
4. Impossibility of recreating peristaltic movements [97], [101], [103]
5. No variation in pH and enzyme activity in each compartment [97], [101], [103];
6. Failure to recreate the gut microbiome [97], [101], [103];

1.9 Scope of the thesis

The aim of this work is to evaluate the bioaccessibility and antioxidant properties of phenolics compounds present in the halophyte plants.

Task 1: Selection of halophyte plants

In this task, 7 halophyte plants (*M. nodiflorum; C. edulis,; I. chritmoides; M. crystallinum; S. ramosissima; S. fructicosa; C. Maritimum*) were screened for their phenolic content and composition in order to select the most promising samples to be evaluated in the *in vitro* digestion protocol. For this prupose, the identification of phenolic compounds by HPLC-DAD-ESI-MS/MS was carried out to 3 plants, namely *M. nodiflorum, M. crystallinum* and *C. edulis* , to complement the previous data of the host lab. Then the quantification of each phenolic compound present in the 7 plants was performed by HPLC-DAD.

Task 2: Bioaccessibility of phenolic compounds

In this task, the two most promising halophyte plants were submitted to an *in vitro* digestion method using the standardized INFOGEST protocol. The digestive fractions, namely oral, gastric and intestinal were characterized in terms of phenolic content and composition (by HPLC-DAD) and anti-oxidant capacity (by complementary tests ORAC and HOSC) aiming to evaluate the bioaccessibility of the main bioactive compounds.

2

MATERIALS AND METHODS

2.1 Chemicals and reagents for *in vitro* digestion

The chemicals and reagents used *in vitro* digestion, sodium bicarbonate, sodium phosphate monobasic, 3,5- dinitro salicylic acid, N-Benzoyl-L-tyrosine ethyl ester, sodium taourodeoxycholate hydrate, tributyrin, Nalpha-p-Tosyl-L-arginine methyl ether hydrochloride, hemoglobin from bovine blood, calcium chloride, pancreatin from porcine gastric mucosa, pepsin from porcine pancreas, bovine serum albumin (BSA), biliar salts, AAPH (2,2-azobis(2- methylpropionamidine)dihydrochloride), Trolox (6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid) and fluorescein sodium salt, all were purchased from Sigma-Aldrich. Tris(hydroxymethyl) aminomethane was purchased from Sial.

2.2 Plants materials and extracts

2.2.1 Plant extracts

Figure 2.1: Images of the seven halophyte plants used in the study. (Images from Riafresh® website: https://www.riafresh.com/index.php/pt/produtos)

Seven species (*Carpobrotus edulis, Crithmum Maritimum, Inula chritmoides, Mesembryanhtemum crystallinum, Mesembryanthemum nodiflorum, Salicornia ramosissima, Sarcocornia fruticosa*) were studied and their extracts were previously prepared according to Oliveira-Alves *et al.* (2021) [57], **Figure 2.1**. The plants were acquired from Riafresh® (Faro, Portugal). The growing and harvesting of these plants were carried out in the 'Parque Natural da Ria Formosa e da Costa Algarvia' in

hydroponic growing conditions. The halophyte plant was stored between 3 °C and 7 °C until further use (for 15 days maximum). All extract presented a concentration of 5 g of plant/1 mL of solvent and were stored at -20 °C.

2.2.2 Selection of plant samples for *in vitro* digestion

According to the first section of the thesis, two of the most promising species were chosen to proceed with digestion studies The two plants selected for the *in vitro* digestion studies*: Salicornia ramosissima and Sarcocornia fructicosa*, were kindly provided by Riafresh ((Faro, Portugal). The production and harvesting of these plants were carried out in the 'Parque Natural da Ria Formosa e da Costa Algarvia' in hydroponic conditions. The halophyte plants were stored between 3 ºC and 7 ºC until futher use (for 15 days maximum).

2.2.3 Freeze-drying processing

The freeze-drying process of the plants took place as follows: first, the fresh plants were frozen using liquid nitrogen (quick freezing), then they were ground (Grounder Moinho Flama, Aveiro, Portugal) and finally placed in the freeze-dryer (ScanVac, Coolsafe 95 /55– 80 freeze dryer, Lynge, Denmark) for 1 day, **Figure 2.2**.

Figure 2.2: *Salicornia ramosissima* after subjected to the freeze dryer process

2.3 Extraction of phenolic compounds

The ultrasonic extraction method was applied to extract the phenolic compounds of the halophyte plants, as described by Oliveira-Alves *et al.* (2021) with some modifications [57]. First, 2 g of the lyophilized plant was added to 100 mL of ethanol: water (80:20, v/v) solution at room temperature, This mixture was shaken at the vortex for a period of time of 10 s and immediately placed in an ultrasonic bath (ArgoLab DU-100, China), The ultrasonic bath was set at five power of potency and maintained at 25 ± 3 ℃ for 60 minutes. After that, the samples were placed in a centrifuge (Sorvall ST16 centrifuge – ThermoFisher Scientific, Germany) and centrifuged for 15 min at 6000 g, The supernatant was collected and was submitted to vacuum filtration. After the filtration, the samples were concentrated in the rotavapor (Büchi R-114, Switzerland) for dryness at ± 40 °C under reduced pressure (until 30 mBar). The obtained residue was resuspended using ethanol: water (50:50, v/v) solution, to obtain a final concentration of 1 g/mL, and filtered using a 0.22 mm SFCA membrane (Branchia, Spain). All the samples were maintained at -20°C until further analysis. The extractions were performed in triplicate.

2.4 *In vitro* digestion

2.4.1 Enzymatic activity determinations

The determination of enzyme activity is a critical step in the process of *in vitro* digestion. For that reason, the activity of the two enzymes/extracts used (gastric pepsin and pancreatin) were performed following the protocols described in the annexes of Brodkord *et al*. (2019) with some adaptations [101].

2.4.1.1 Pepsin activity determination

For the determination of the pancreatin activity, 500 µL of hemoglobin solution (substrate) were pipetted to each tube and incubated at 37ºC for 4 minutes. Then 100 µL of each prepared enzyme concentration (5, 10, 15, 20, 25, 30 and 35 µg/mL) were added and incubated for 10 minutes at 37ºC in the ultrasonic bath (ArgoLab DU-100, China). After, 1 mL of 5 % TCA (trichloroacetic acid) was added to stop the enzymatic reaction. All the tubes were centrifuged in the Sorvall ST16 centrifuge (ThermoFisher Scientific, Germany) for 30 minutes at 6000 g. The supernatant was collected, placed in a quartz cuvette, and read at 280 nm.

2.4.1.2 Pancreatin activity determination

For the determination of pancreatin activity, the trypsin assay was used. In this assay, 2.6 mL of Tris-HCl buffer and 300 µL of TAME (Nα-p-Tosyl-L-arginine methyl ester) were placed in the cuvette, mix, and let incubate at 25ºC for about 4 minutes. Then 100 µL of pancreatin solution was added to the cuvette and the absorption was read at 245 nm and recorded for 10 minutes in the spectrophotometer (Ultrospec 3000, Pharmacia Biotech). This procedure was repeated for all the pancreatin solutions of different tested concentrations (0.25; 0.5 and 1 mg/mL)

2.4.2 Preparation of stock solution of simulated digestion fluid

The preparations of stock solutions of electrolyte solutions required to obtain a final volume of 0.4 L of simulated digestion fluids at a 1.25× concentration were performed according to **Table 2.1**:

Table 2.1: Stock solutions of salt solutions, adapted from Brodkord (2019) *et al.* [101]

Solution	Concentration (M)	Weight(g)	Volume of water (mL)
$CaCl_2(H_2O)_2$	0.30	2.2050	50
KCl	0.50	7.4560	200
KH_2PO_4	0.50	6.8040	100
$NaHCO_3$	1.00	16.8020	200
NaCl	2.00	23.3780	200
$MgCl_2(H_2O)_6$	0.15	1.5250	50
$(NH_4)_2CO_3$	0.50	2.4025	50

Solutions of NaOH (1M) and HCl (1M and 6M) were also prepared for pH adjustment. Then these stock solutions of electrolyte solution were mixed according to **Table 2.2** to obtain the three stock solutions of simulated digestion fluids: Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), Simulated Intestinal Fluid (SIF).

Table 2.2: Stock solutions of simulated digestion fluids, adapted from Brodkord *et al.* (2019); * Mililiters of stock added to prepare 400 mL (x1.25) [101]

Salt solution added	Stock concentrations		SSF		SGF		SIF	
	g/L	M	V (mL)*	Final salt concentration in the fluid: (mM)	V (mL)*	Final salt concentration in the fluid:	V (mL)*	Final salt concentration in the fluid:
KCl	37.3	0.50	15.100	15.10	6.900	6.90	6.80	6.80
KH_2PO_4	68.0	0.50	3.700	3.70	0.900	0.90	0.80	0.80
$NaHCO_3$	84.0	1.00	6.800	13.60	12.500	25.00	42.50	85.00
NaCl	117.0	2.00	–	–	11.800	47.20	9.60	38.40
$MgCl_2(H_2O)_6$	30.5	0.15	0.500	0.15	0.400	0.12	1.10	0.33
$(NH_4)_2CO_3$	48.0	0.50	0.060	0.06	0.500	0.50	–	–
HCl	–	6.00	0.090	1.10	1.300	15.60	0.70	8.40
$CaCl_2(H_2O)_2$	44.1	0.30	0.025	1.50	0.005	0.15	0.04	0.60

The stock digestion fluids were stored at -20 °C until further use. On the day of the *in vitro* digestion assay, the solutions were defrosted at 37 ºC and the Ca^{2+} solution, bile salts, enzymes, and water were added to obtain the correct electrolyte concentration (1x).

2.4.3 *In vitro* digestion assay of plants

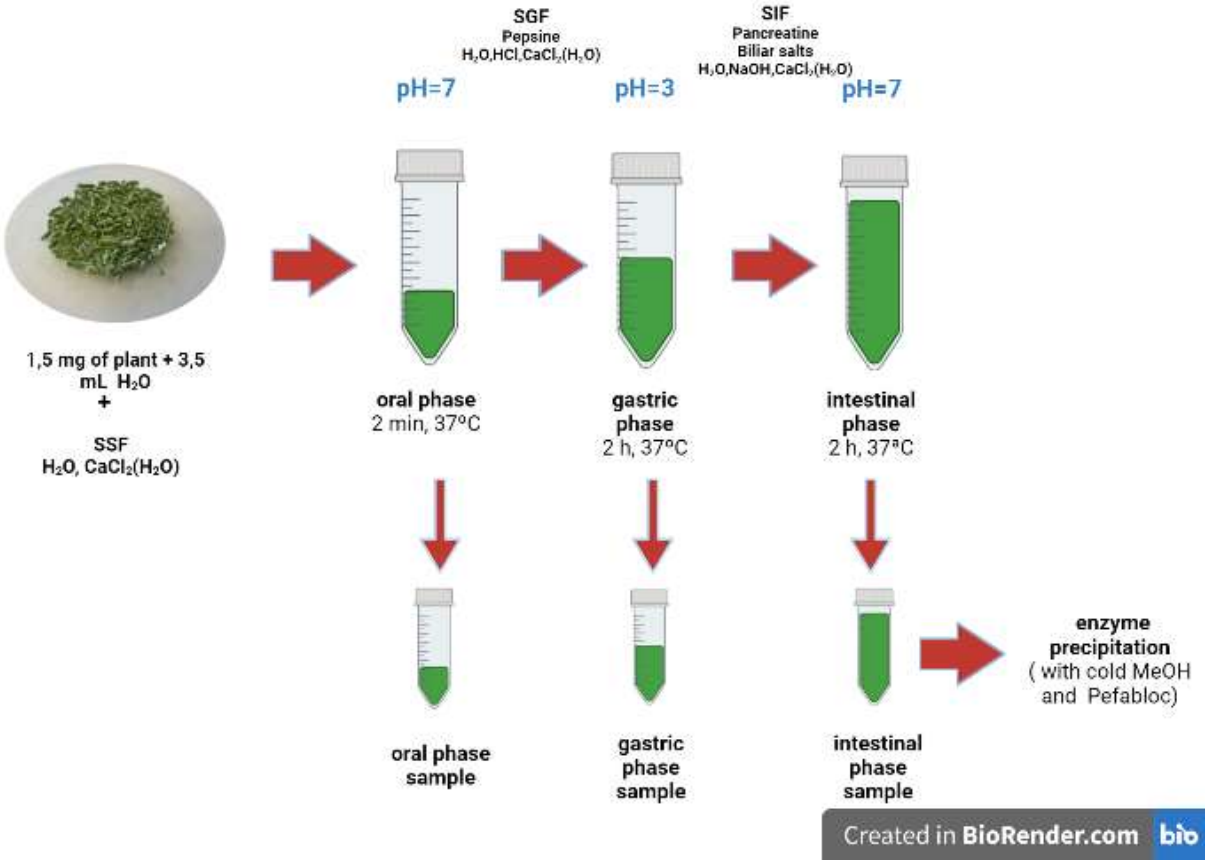

Figure 2.2: Diagram of the *in vitro* digestion procedure applied to *Salicornia ramosissima* and *Sarcocornia fruticosa*

Samples were digested according to the INFOGEST® *in vitro* gastrointestinal methodology employing the previously prepared simulated saliva fluid (SSF), simulated gastric fluid, (SGF), and simulated intestinal fluid (SIF) [101]. After the determination of enzymes' activity, the correct weight of enzymes was measured to obtain the desired final concentration in the respective fluid. All the enzymes were prepared daily, **Figure 2.2**.

2.4.3.1 Oral phase

In the oral phase, 5 mL solution of halophyte plant (1.5 mg of freeze-dried plant + 3.5 mL of water) were combined with 25 µL of 0.3 M $CaCl_2$, 4 mL of Simulated Salivary Fluid (at 1.25X concentration, pH 7), and 0.975 mL of MiliQ water to obtain a final volume of 10 mL. The mixture was incubated for 2 minutes in a water bath at 37 °C with constant stirring.

2.4.3.2 Gastric phase

To simulate gastric digestion, the oral phase mixture was transferred to the gastric phase by adding 5 µL of 0,3 M $CaCl_2$, 8 mL of gastric fluid (at 1.25 x concentration, pH 3), and 1 mL of pepsin solution (EC 3.4.23.1; Sigma, USA). The pH was brought to pH 3.0 using a solution of 1M HCl. To

obtain a final volume of 20 mL, MiliQ water was added to the mixture. The solution was incubated for 120 min at 37 °C in the water bath with constant agitation.

2.4.3.3 Intestinal phase

The mixture resulting from gastric digestion was transferred to the intestinal phase. In the mixture resulting of gastric digestion 40 µL of 0.3 M $CaCl_2$, 8.5 mL of SIF (at concentration 1,25x , pH 7), 5 mL of pancreatin solution (Sigma, USA) and 2.5 mL of bile extract porcine (Sigma, USA) were added. A solution of 1M of NaOH was used to raise the pH to 7 and the final volume was adjusted to 40 mL by adding MiliQ water. The solution was maintained in the water bath at 37°C for 120 min with constant stirring.

At the end of each simulated digestion phase, the tubes containing the samples were collected. Then, these samples were frozen with liquid nitrogen and stored at -80ºC until further analysis. Before LC analysis, the enzymes were precipitated by diluting the collected samples with pure methanol (1:1). Then samples were centrifuged at 10 000 g for 15 min at 4°C. The samples were placed in the rotavapor (Büchi R-114, Switzerland) until the total dryness of methanol. The solution was resuspended in water to obtain a final concentration of 0.1 g/mL and analyzed by HPLC-DAD-MS/ MS [104], [105].

2.5 Characterization of samples

2.5.1 Phytochemical characterization

2.5.1.1 Total phenolic content estimation by Folin Ciocalteu assay

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) of the phenolic extracts and digestive fractions from halophyte plants. The method used was described by Singleton *et al. (1965)* with some adaptation for microplate [106]. Briefly, a reaction mixture containing 230 µL of milli-Q water, 15 µL of Folin-Ciocalteu's reagent, and 10 µL of the extract/standard was placed in each well and incubated for 3 minutes at room temperature. After this period, 45 µL of a solution at 35% of Na_2CO_3 was placed in each well, and the microplate was incubated at room temperature for 1 h without exposure to light. The absorbance was recorded at 765 nm in a microplate reader (Epoch2, Biotek, Winooski, VT, USA) using Gen5 3.02 software for data acquisition and processing.

Gallic acid concentrations (7.5, 15, 30, 120, 180, and 240 µM) were prepared to obtain a standard curve. The results obtained were expressed in gallic acid equivalents by using the standard curve calculated (mg GAE/g). All experiments were performed in duplicate at room temperature.

2.5.1.2 Quantification and identification of phenolic compounds by liquid chromatography (LC) with diode array detector (DAD)

The analyses of phenolic compounds were performed as described by Oliveira-Alves *et. al* (2021) [57]. The halophyte plants' extracts and digestive fractions were analyzed in the Thermo Dionex Ultimate (Thermo Fisher Scientific, USA) equipment fitted with a pump, auto-sampler, and photodiode

array detector (Thermo Dionex DAD-300). The samples were separated chromatographically using a Luna C18 reversed-phase (Luna 5 µm C18(2) 100 Å, 250 x 4 mm; Phenomenex) which was preceded by a Manu-cart RP-18 pre-column set at 35 °C. DAD has performed a scan range between 192 and 798 nm with a frequency of 1 Hz and a bandwidth of 5 nm. The injection volume was 10 µl and the auto sampler's temperature was set at 12ºC. The mobile phase A was made up of water: formic acid (99.5%:0.5%), and the mobile phase B, of acetonitrile (100%). The gradient program was the following: 0-10 min from 99 to 95% A; 10-30 min, from 95 to 82% A; 20-44 min, from 82 to 64% A; 44- 64 min at 64% A; 64-90min from 64 at 10% A; 90-100 min at 10%; 100-101 min, from 10 to 95% A; from 101-120 min the phase conditions were reset to the initial conditions. The eluents A and B were applied at a flow rate of 0.30 mL/min. DAD proceeded at three different wavelength: 280, 320, and 360 nm with a frequency of 10 Hz and a bandwidth of 11 nm. Comparation with commercial standards (gallic acid, isorhamnetin, quercetin-3-glucoside and chlorogenic acid) retention time and the UV-Vis Spectrum were used for compounds' identification. The quantification was made after analysis of the corresponding compounds' calibration curves.

2.5.2 Antioxidant activity

2.5.2.1 ORAC Assay

The antioxidant capacity of the samples against peroxyl radicals was measured using the ORAC assay as described by Serra *et al.* (2011) [107]. Briefly, 25 µL of the sample was mixed with 150 mL fluorescein ($3x10^{-4}$ mM concentration) solution and placed in the well of a 96-well microplate. On the FL800 microplate fluorescence reader, the mixture was pre-incubated for 10 minutes at 37°C (FL800 Bio-Tek Instruments, Winooski, VT, USA). Then, using the plate reader dispenser, 25 µL of AAPH solution (12 mM final concentration) was quickly injected into each well, and the fluorescence was registered every minute for 40 minutes.

All the reaction mixtures were made in duplicate for each sample. A standard curve was prepared to determine ORAC values in umol of trolox equivalents (TEAC)/ g of sample.

2.5.2.2 HOSC Assay

The hydroxyl radical scavenging capacity of halophytes and digested extracts were estimated using the HOSC assay, as described by Moore *et al.* (2006) with some modifications [108]. The assay reactions were carried out in the FL800 microplate fluorescence reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). The reaction mixture contained 170 µL of fluorescein solution, 30 µL of blank/Trolox /sample, 40 µL of H_2O_2, and 60 µL of $FeCl_3$, added in that order. The plate was read in each well once per minute for 1 h. Trolox concentrations of 0, 10, 15, 20, and 25 µM were used for the calibration curve. HOSC values were calculated using the regression equation. HOSC values were expressed as µmol Trolox equivalents (TEAC) /g of sample.

3

RESULTS AND DISCUSSION

3.1 Selection of halophyte plants

Currently, there is few information available about the phenolic content of the halophyte plants under study, namely *Carpobrotus edulis, Crithmum Maritimum , Inula chritmoides, Mesembryanhtemum crystallinum, Mesembryanthemum nodiflorum, Salicornia ramosissima and Sarcocornia fructicosa*. In a previous project at iBET (Fábio Andrade's master's work (2021)) "Study of halophyte plants produced in Portugal"), the identification of phenolic compounds in 4 species of halophyte plants, namely *Inula Chritmoides, Chritmum maritimum, Salicornia ramosissima and Sarcocornia fruticosa,* was performed by LC-DAD-ESI-MS/MS with ESI negative ionization mode [109]. It was demonstrated that:

- In Inula (*Inula chritmoides*): 5 organic acids (quinic acid derivative, quinic acid and quinic acid derivative, cinnamic acid, and malic acid) , 14 phenolic acids (protocatechuic acid-glucoside, 2 gallic acid derivative, syringic acid, 2 caffeic acid derivative, p-coumaric acid, p-coumaric acid derivative, caffeic acid-O-glucoside, 5-caffeoylquinic acid, feruloyquinic acid, piscidic acid, caffeic acid-glucoronide-glucoside) and 5 flavonoids (rhamnetin,gallocatechin, pinobanksin-5-methyl-ether-3-O-acetate, gallocatechin derivative and vitexin);
- In Sea fennel (*Crithmum maritimum*): 3 organic acids (quinic acid, malic acid and citric acid), 9 phenolic acids (protocatechuic acid-glucoside, caffeic acid-O-glucoside, p-coumaric acid-glucoside , p-coumaroylquinic acid (isomer 1 and isomer 2), 2 p-coumaric acid derivative, 3-caffeoylquinic acid and 3,5-O-dicaffeoyquinic acid) and 6 flavonoids (pinobanksin-3-O-pentanoate,apigenin 6-C-glucoside-7-O-glucoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, apigenin 6-C-glucoside and diosmedin 7-O-rutinoside)

- In Salicornia (*Salicornia ramossisima*): 4 organic acids (quinic acid derivative, quinic acid, malic acid and succinic acid), 14 phenolic acids (3-O-caffeoylquinic acid, p-coumaric acid, p-coumaric acid derivative, 5-caffeoylquinic acid, ferulic acid, ferulic acid-glucoside, protocatechuic acid , p-coumaroylquinic acid (isomer 1 and 2), ferulic acid derivative, 4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid and caffeoylhydrocaffeoylquinic acid) and 3 flavonoids (quercetin 3 -O-hexoside,Isorhamnetin-3-O-glucoside and gallocatechin);
- In Sarcocornia (*Sarcocornia fruticosa*): 5 organic acids (quinic acid derivative, malic acid, quinic acid, quinic acid derivative and succinic acid) , 14 phenolic acids (5 p-coumaric acid derivative, 5-galloylquinic acid, 5-O-caffeoylquinic acid, protocatechuic acid-arabinoside, 2 caffeic acid derivative,3-caffeoylquinic acid, caffeoylquinic acid derivative, p-coumaroylquinic acid and 3-O-coumaroyl-5-O-caffeoylquinic acid) and 6 flavonoids (gallocatechin, dihydroquercetin, eriodyctiol-O-hexoside, epicatechin-pentose, rhamnetin hexosyl pentoside and isorhamnetin-3-O-robinobioside)

To complement, this study, 3 more plants were analyzed regarding their phenolic content: Ice plant (*Mesembryanhtemum crystallinum*), Slenderleaf Ice Plant (*Mesembryanthemum nodiflorum*) and Sea fingers (*Carpobrotus edulis*). The tentative identification of phenolic compounds was based on maximum UV absorption, retention time (t_R), precursor ion and correspondent MS/MS fragment ions, and supported by bibliographic references [57].

3.1.1 Identification of phenolic compounds present in *Mesembryanthemum crystallinum, Mesembryanthemum nodiflorum and Carpobrotus edulis*

Ice plant (*Mesembryanthemum crystallinum*)

In Ice plant, 17 phenolic compounds and 5 organic acids (cinnamic acid, quinic acid, quinic acid derivative, malic acid, and cinnamic acid derivative) were identified. From 17 phenolic compounds: 8 compounds were flavonoids (gallocatechin, epigallocatechin, pinobanksin-2-pentaonate, kaempferol derivative, acacetin, 3,6-di-glucoside, chrysin-6-C-glucoside-8-C-arabinoside, quecetin-3-O-glucoside, 2-rhamnosyl-2-glucosyl-kaempferol derivative) and 7 of them were phenolic acids (p-coumaric acid-O-glucoside, p-coumaryolquinic acid, ferulic acid-O-glucoside, ferulic acid derivative, feruloylglucaric acid, p-coumaric acid derivative, caffeoylsinapylquinic acid, and p-coumaric acid glucoside derivative), **Table 3.1**.

Table 3.1: Identification and quantification of phenolic compounds of Ice Plant (*Mesembryanhtemum crystallinum***) using mass spectrometry *** concentration expressed in µg/g FW; The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalent

Peak	R_T (min)	λmax (nm)	$[M-H]^-$ m/z	HPLC–DAD–ESI-MS/MS m/z (% base peak)	Tentative Identification	Refe-rences	Concentration, average ±SD*
1	7.35	276	147	103(20); 120(10); 62(100)	cinnamic acid	[110]	
2	8.22	256	133	115(30); 71(100); 89(40); 113(10)	malic acid	[111], [112]	
3	9.37	261	191	111(60); 173(10); 171(10); 155(10); 127(10); 109(10)	quinic acid	[113], [114]	
4	9.77	260	243	191(70); 111(80); 173(10)	quinic acid derivative	[113], [114]	
5	11.65	258	227	147(40); 62(100); 120(10); 103(10)	cinnamic acid derivative	[110]	
6	34.08	275; 319	305	219(10); 179(20)	gallocatechin	[115]	0.18 ± 0.02
7	36.15	274; 325	325	163(20); 119(50)	p-coumaric acid-O-glucoside	[116]	0.25 ± 0.09
8	36.40	294	337	191(10); 173(20); 163(20)	p-coumaroylquinic acid (isomer 1)	[117]	0.62 ± 0.08
9	36.98	276; 310	305	219(10); 179(10); 221(10); 261(10)	epigallocatechin	[115], [118]	0.52 ± 0.05
10	37.80	277	355	173(100)	ferulic acid-O-glucoside	[112], [112]	0.50 ± 0.06
11	38.40	328	355	253(50); 181(60); 165(10); 143(10); 107(20)	pinobanksin-3-O-pentanoate	[119]	1.57 ± 0.27
12	39.30	280; 319	355	193(20); 178(40); 135(20)	ferulic acid derivative	[120]	0.77 ± 0.07
13	39.72	280	385	223(100); 208(40); 164(40)	sinapic acid -glucoside	[113], [121]	3.35 ± 0.36
14	40.38	269; 330	385	191(10); 173(50)	feruloylglucaric acid	[122]	0.51 ± 0.06
15	41.23	277; 324	433	417(40); 285(60); 229(50); 151(40)	kaempferol derivative	[123]	0.58 ± 0.01
16	45.88	270; 333	607	487(30)	acacetin 3,6-di-C-glucoside	[124]	0.45 ± 0.04
17	46.30	282; 311	547	529(10); 337(10); 367(20)	chrysin-6-C-glucosyl-8-C-arabinoside	[125]	7.36 ± 0.48

Table 3.1: Identification and quantification of phenolic compounds of Ice Plant (*Mesembryanhtemum crystallinum***) using mass spectrometry *** concentration expressed in µg/g FW; The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalent **(cont.)**

Peak	R_T (min)	λmax (nm)	$[M-H]^-$ m/z	HPLC–DAD–ESI-MS/MS m/z (% base peak)	Tentative Identification	Refe-rences	Concentration, average ±SD*
18	47.12	314	581	163(100); 119(10)	p-coumaric acid derivative	[126]	14.49 ± 0.02
19	47.65	311	559	163(40)	caffeoylsinapylquinic acid	[127]	17.15 ± 0.18
20	48.50	319	589	325(60); 163(10)	p-coumaric acid glucoside derivative	[128]	0.14 ±0.01
21	49.60	272; 354	575	463(15); 300(80); 301(40); 179(20); 151(10)	quercetin-3-O-glucoside derivative	[58]	0.69 ± 0.07
22	52.88	285	901	739(40); 593(85); 285(10)	2-rhamnosyl–2-glucosyl kaempferol	[116], [129]	0.59 ± 0.06

Organic acids have been found among the compounds identified in the Ice Plant extracts. Peak 1 corresponds to cinnamic acid with precursor ions $[M-H]^-$ at m/z 147 and product ions at m/z 120, 103 and 62 [110]. Also, the peak 5 with precursor ions $[M-H]^-$ at m/z 227 and product ions at m/z 120, 103 147 and 62 were tentatively identified as a cinnamic acid derivative [110]. The peak 2 was identified as malic acid with precursor ions $[M-H]^-$ at m/z 133 and fragment ions at m/z 115, 113, 89, and 71. The fragment ion at m/z 115, corresponds to a loss of $[M-H-H_2O]^-$, and the fragment ion m/z 71, corresponds to a loss of $[M-H-H_2O-CO_2]^-$ [111], [112]. The quinic acid was also identified as corresponding to peak 3 with precursor ions $[M-H]^-$ at m/z 191 and product ions at m/z 111, 173, 171, 155, 127 and 109. Compound 4, because of its product ions at m/z 111, 173 and 179, was tentatively identified as a compound derivate from quinic acid [113], [114].

The peak 6 corresponds to gallocatechin presenting a precursor ion $[M-H]^-$ at m/z 305 and product ions at m/z 219 and 179 [115]. Gallocatechin is a flavanol. These group of flavonoid compounds are more frequently found in fruits and derived products but also occur in cereals, black and green tea, red wine, and chocolate [130], [131]. Flavo-3-nols are commonly used in cancer treatments including breast cancer treatment [130]. The peak 9 corresponds to epigallocatechin with precursor ions $[M-H]-$ at m/z 305 and fragment ions at m/z 219, 179, 221, and 261. The fragment ions of epigallocatechin m/z 261 result of a loss of one CO_2, m/z 221 correspond to a loss of one $C_4H_4O_2$, m/z 219 correspond to a loss of one $C_4H_6O_2$ and m/z 179 results of a loss of one $C_6H_6O_3$ [118].

The peak 7 corresponds to p-coumaric acid-O-glucoside with parent ion $[M-H]^-$ at m/z 325 and daughter ions at m/z 163 and 119 [132]. p- Coumaric acid-o-glucoside is a conjugate compound of p-coumaric acid. The p-coumaric acid and its conjugates are widely found in fruits, such as berries, tomatoes, oranges, grapes and apples, vegetables, such as beans, onions and potatoes, and cereals such as wheat, oats and maize [133]. Furthermore, p-coumaric acid and its conjugates have several beneficial efects on health, being reported to reduce low density lipoprotein (LDL) oxidation [134]. It also presented anti-bacterial activity by acting as quorum sensing inhibitors [135]. The peak 20 corresponds to p-coumaric acid glucoside derivative with precursor ions $[M-H]^-$ at m/z 589 and product ions at m/z 325 and 163 [128]. The p-coumaric acid glucoside is a bioactive compound also detected in sainfoin extracts and olive pomace [136], [137]. The p-coumaric acid glucoside was also present methanolic extract of *Geranium molle,* an extract that exhibited high cytotoxic properties towards the MCF-7 cancer cell line [128].

The peak 8 corresponds to p-coumaryolquinic acid isomer with precursor ions $[M-H]^-$ at m/z 337 and product ions at m/z 191,173 and 163 [117]. p-Coumaryolquinic acid belongs to the hydroxycinnamic acid class which is a family of esters produced between quinic acid and one or more residues of trans-cinnamic acid such as caffeic, ferulic, or p-coumaric acid [138]. The p-coumaroylquinic acid isomers compounds are also found in the herbal *Chrysanthemum*, in craft beers and sweet cherries [138]–[140].

The peak 10 corresponds to ferulic acid-O-glucoside with precursor ions $[M-H]^-$ at m/z 355 and product ions at m/z 193 [132]. The peak 12 corresponds to ferulic acid derivative precursor ions $[M-H]^-$ at m/z 355 and product ions at m/z 193, 178, and 135 [120]. Some studies proved that ferulic acid had

antihypertensive and anticancer properties. A single dose (9.5 mg/kg of body weight) of ferulic acid in rats (in the hypertensive model), showed effects on the blood pressure, with a significant antihypertensive effect 2 hours after oral administration [141]. Staniforth *et al.* (2011) proved that ferulic acid inhibits the production of matrix metalloproteinases MMP-2 and -9, two proteins which overexpression could result in the appearance of skin cancer [142].

The peak 11 corresponds to pinobanksin-3-pentaonate with precursor ion $[M-H]^-$ at m/z 355 and product ions at m/z 193, 178 and 135 [119]. Pinobanksin-3-pentaonate is a flavonoid present in extracts of propolis. This compounds together with other compounds presented in the extract of propolis presented a great antiparasitic activity against three protozoan belonging to genus *Leishmania* [143].

The peak 13 corresponds to sinapic acid-glucoside with molecular ion $[M-H]^-$ at m/z 385 and product ions at m/z 223, 208 and 164 [113], [121]. Due to the product ion at m/z 223, corresponding to [M–H-hexose], this compound was recognized as a cinnamoyl hexoside (sinapic acid) [121]. The sinapic acid- glucoside is one of the phenolic compounds present in the broccolis, which are proved to possess anticancer and antioxidant activities [144].

The fragmentation pattern of peak 14 with molecular ion $[M-H]-$ at m/z 385 and product ions at m/z 191 and 173 matches the reported in the literature for feruloylglucaric acid [122].

The peak 15 corresponds to kaempferol derivative with precursor ions $[M-H]-$ at m/z 433 and product ions at m/z 417, 285, 229, and 151 [123]. The kaempferol has some proven anticancer effects. Pancreatic, lung, gastric, ovarian, breast, and blood cancers are some of the cancers type where this kaempferol effect has already been demonstrated [145]. The peak 22 corresponds to 2-Rhamnosyl–2-glucosyl kaempferol with precursor ions $[M-H]^-$ at m/z 901 and product ions at m/z 739, 593, and 285. Fragmentation of the m/z 901 ion results in a product ion at m/z 739 resulting from the loss of 162 Da, which represents the elimination of a glycosyl group [129]. A loss of 146 Da results in the product ion m/z 593, which indicates the removal of a rhamnosyl group [129]. The precursor ion m/z 593 corresponds to rhamnosyl–glucosyl kaempferol and the m/z product ion 285 is obtained throw a loss of 308 Da which is a result of the elimination of a rhamnosyl–glucosyl moiety [129]. The m/z 285 is characteristic of kaempferol.

The peak 16 corresponds to acacetin 3,6-di-C-glucoside with precursor ions $[M-H]^-$ at m/z 607 and product ions at m/z 487. Di-C-glycosylflavones are distinguished by the lack of the aglycone ion and the presence of the product ion at m/z 487 for $[M-H-120]^-$. The absorbance in the UV-VIS spectrum together with the MS fragmentation pattern, allowed the identification of the compound [124].

The peak 17 correspond to chrysin-6-C-glucoside-8-C-arabinoside with precursor ions $[M-H]-$ at m/z 547 and product ions at m/z 529, 337 and 367 [125]. The chrysin-6-C-glucoside-8-C-arabinoside was detected in a traditional Chinese herb based formula that researchers proved to have the capacity to inhibit SARS-CoV-2 pathogenesis [146].

The peak 18 corresponds to p-coumaric acid derivative with precursor ions $[M-H]-$ at m/z 581 and product ions at m/z 163 and 119 [126].

The peak 19 corresponds to caffeoylsinapylquinic acid with precursor ions $[M-H]-$ at m/z 559 and product ions at m/z 163 [127]. The caffeoylsinapylquinic acid was the main compound present in

Tunisian date syrup. Extracts of this syrup have demonstrated strong antioxidant activities and also antibacterial effect against a variety of bacterial strains [147].

The peak 21 correspond to quercetin-3-O-glucoside derivative with precursor ions [M−H]− at m/z 575 and product ions at m/z 463, 300, 301, 179 and 151 [58]. Quercetin 3- o-glucoside had some antioxidant and antidiabetic effect. Quercetin-3-O-glucoside extracted from the shoot of *Prangos ferulaceae* displayed high antioxidant activity having a IC_{50} value of 22 µg/mL in DPPH assay [148]. Also, quercetin-3-O-glucoside isolated from *Annona squamosa* leaves appeared to have antidiabetic effect by stimulation of the insulin [149].

Slenderleaf Ice Plant (*Mesembryanthemum nodiflorum*)

In Slenderleaf Ice Plant, 5 organic acids (citramalic acid, malic acid, quinic acid cinnamic acid and succinic acid) and 25 phenolic compounds were identified. From the 25 phenolic compounds, 12 are phenolic acids (2 ferulic acid derivative, p-coumaric-O-glucoside, ferulic acid-glucoside, syringic acid derivative, p-coumaric acid derivative, digalloyl quinic acid rhamnoside, hexahydroxyphenoyl-glucose, caffeoylquinic acid derivative, 2 galloylquinic acid derivative and 3,5-diferuoylquinic aid) and 13 flavonoids (2 pinocembrin derivative, gallocatechin, epigallocatechin, avicularin,chysin-6-C-glucoside-8-C-arabinoside,eriodyctiol-O-hexoside, eriodyctiol, epicatechin, quercetin dipentoside, quercetin derivative, vitexin derivative, and pinobanksin-5-methyl ether-3-O-acetate), **Table 3.2.**

The peak 1 was identified as citramalic acid with precursor ion [M−H]− at m/z 147 and product ions at m/z 129, 103, 87 and 85. The mass transition of m/z 147 to m/z 87 results of a loss of $[M\text{-}HCH_3COOH]^-$ [149]. The peak 2 corresponds to malic acid with molecular ion $[M-H]^-$ at m/z 133 and product ions at m/z 115, 89 and 71 [112], [113]. The peak 3 was identified as quinic acid with precursor ion $[M-H]^-$ at m/z 191 and product ions at m/z 87, 111 and 85 [114], [115]. The peak 4 corresponds to cinnamic acid with precursor ion $[M-H]^-$ at m/z 147 and product ions at m/z 103 and 62 [111]. The peak 5 was characterized as succinic acid presenting a precursor ion [M−H]− at m/z 117 with fragment ions at m/z 99 and 73. The fragment ion 73 corresponds to $[M-H-CO_2]^-$ [113].

The peak 6 was tentatively identified as pinocembrin derivative presenting precursor ion $[M-H]^-$ at m/z 323 with product ions 255, 213, 211 and 237 [150]. This peak was identified as a pinocembrin derivative because it showed the presence of MS/MS fragmentation ions in m/z 255 (pinocembrin) but also fragmentation ions characteristic of pinocembrin as ion 237 that corresponds to the loss of $[M\text{-}H\text{-}OH]^-$, ion m/z 213 corresponding to the loss of $[M\text{-}H\text{-}C_2H_2O]^-$, and ion m/z 211 resulting from the elimination of $[M\text{-}H\text{-}CO_2]^-$ [150]. Pinocembrin isolated from ethanolic extracts of *Alpinia price* presented an anti-inflammatory effect by suppression of lipopolysaccharide-stimulated prostaglandin E_2 and nitric oxide production [151]. Also investigation of pinocembrin present in chloroformed extract of a desert plant, *Centaurea eryngioides*, indicated a potential antitumor capacity [152]. The peak 14 also was characterized as pinocembrin derivative presenting a precursor ion $[M-H]^-$ at m/z 387 and product ions at m/z 255, 211, 213 and 151 [150].

The peak 7 correspond to ferulic acid-glucoside derivative with precursor ion $[M-H]^-$ at m/z 553 and product ions at m/z 355, 193, 155, 134 and 178 [132]. The peak 11 was characterized as ferulic

acid-glucoside with precursor ion $[M-H]^-$ at m/z 355 and product ions at 193,178,149 and 134 [132]. The peak 12 correspond to a ferulic acid derivative with precursor ion $[M-H]^-$ at m/z 321 and product ions at 193 and 119 [132].

The peak 8 had a molecular ion $[M-H]^-$ at m/z 305 and fragment ions at 225, 208 and 97, which is typical of gallocatechin [153]. The peak 10 corresponds to epigallocatechin with precursor ion $[M-H]^-$ at m/z 305 and product ions 225, 208 and 97. Both gallocatechin and epigallocatechin exhibited the same mass spectrum and the same fragmentation pattern, being distinguished only by their retention time [154], [155]. The gallocatehin and epigallocatechin showed some promotive effect in bone remodulation and metabolism [151]. The peak 20 was tentatively identified as epicatechin derivative with precursor ion $[M-H]^-$ at m/z 307 and products ions at 289, 245, 179 and 205 [154].

The peak 9 was tentatively characterized as p-coumaric-o-glucoside with precursor ion $[M-H]^-$ at m/z 325 and product ions at 163 and 119 [116]. The p-coumaric-o-glucoside, was found to be one of the major compounds present in the leaves of cowpeas cultivars [157]. Moloto *et al.* (2020) showed an antidiabetic effect of p-coumaric-O-glucoside, by proving a positive correlation between the presence of this compound and the inhibition of the α-glucosidase and α-amylase, two enzymes associated with carbohydrate digestion [157]. The peak 16 correspond to the p-coumaric acid derivative with precursor ion $[M-H]^-$ at m/z 391 and product ions at 337 and 163 [126].

The peak 13 had a precursor ion $[M-H]^-$ at m/z 433 and fragment ions at 271, 301 and 151, which is reported in the literature to be avicularin [158], [159]. Avicularin proved to have a protective effect in rheumatoid arthritis *in vitro* model by lowering inflammatory markers such as in metalloproteinase MMP-1 and interleukin 6 [160]. Avicularin also present potential anticancer effect. This compound reduces the drug resistance of human gastric cancer cells to cisplatin, a compound used in the treatment of this type of cancer. The combination of avicularin and cisplatin decreased tumor cell proliferation and triggered apoptosis [161].

The peak 15 was characterized as syringic acid derivative presenting a precursor ion $[M-H]^-$ at m/z 423 and product ions at 197, 182, 167, 152 and 125 [162]. Syringic acid occurs in high abundancy in some food matrices such as red wine, honey, grapes, dates, spices, pumpkins and olives [163]. Syringic acid could act as chemotherapeutic agent in treatment of gastric cell cancer by suppressing the inflammation and proliferation of cancer cell and triggering the apoptosis [159].

The peak 17 was identified as Chrysin-6-C-glucoside-8-C-arabinoside with precursor ion $[M-H]^-$ at m/z 547 and product ions at m/z 487, 529, 457, 427, 367 and 337 [125].

The peak 18 correspond to eriodyctiol-O-hexoside with precursor ion $[M-H]^-$ at m/z 449 and product ion at 287. The nature and position of the sugar residue could not be established. This compound is generally present in the herbal plant thyme (genus *Thymus*) [165]. The peak 19 correspond to eriodyctiol with precursor ion $[M-H]^-$ at m/z 287 and product ions at 135, 151 and 107 [166]. The eriodyctiol is the most abundant flavonoid presented in a large number of medicinal plants, vegetables and citrus fruits [167]. Eriodyctiol proved to have some antidiabetic activity by promoting insulin-stimulated glucose uptake [168].

The peak 21 correspond to digalloyl quinic acid derivative with precursor ion $[M-H]^-$ at m/z at 641 and products ions at 495 and 191 [169].

The peak 22 was identified as quercetin dipentoside because it had molecular ion $[M-H]^-$ at m/z at 565 and products ions at 301, 300, 179 and 151 [170]. The fragment ion 301 correspond to the quercetin aglycone (loss of 264 mass units due to lose of two pentoses) [171]. The peak 23 corresponds to quercetin derivative having a parent ion $[M-H]^-$ at m/z at 415 and products ions at 300 and 301 [172]. Quercetin is one of the most prevalent flavonoids in fruits and vegetables, occurring essentially as aglycone or glycosides form [173]. Apples, French beans, broccoli, lettuce, onions and tomatoes are some examples of vegetables that have a high concentration of quercetin [173], [174]. Quercetin induces a favorable antioxidant effect on the human hepatoma cell line (HepG2) by reducing the concentration of MDA (malondialdehyde) and the production of ROS [175]. Quercetin could also have anticancer property by preventing the angiogenesis of tumors [176].

The peak 24 was tentatively identified as hexahydroxydiphenoyl-glucose presenting a molecular ion $[M-H]^-$ at m/z at 481 and fragments ions at 301 and 275 [177], [178]. The hexahydroxydiphenoyl-glucose extracted from the peel of *Punica granatum*, exhibited high radical quenching and antioxidant potential [179].

Peak 25 is a caffeoylquinic acid derivative with a precursor ion $[M-H]^-$ at 565 m/z and products ions at 353, 179, 111 and 191 [180]. The caffeoylquinic acids are esters of caffeic acid with quinic acid [181]. The formation of these metabolites occurs in the phenylpropanoid biosynthesis pathway [181]. A caffeoylquinic acid derivative, a 3,4,5-tricaffeoylquinic acid , promotes an improvement of memory and spatial learning by having pro-neurogenic activity in the hippocampus [182]. Also, caffeoylquinic acid derivatives obtained from *Moringa oleifera* leaves extracts, showed to have an inhibition effect against four bacterial strains tested (*B. cereus, S. aureus, S. typhimurium and E. coli*) [183]. The 5-O-Caffeoylquinic acid was the major compound present *in Ptychotis verticillata* infusion. This infusion showed antibacterial activity against ten bacterial strains (*Morganella morganii,* Methicillin-Sensitive *Staphylococcus aureus* (MSSA), *Pseudomona aeruginosa,* Methicillin Resistant *Staphylococcus aureus, Escherichia Coli, Listeria monocytogenes, Klebsiella pneumoniae,* Extended Spectrum Beta-Lactamase (ESBL)-producing *E. coli, Enterococcus faecalis* and Extended-Spectrum Beta- Lactamase (ESBL)-producing *K. pneumoniae*), including multi-resistant strains (Methicillin Resistant *Staphylococcus aureus*, Extended Spectrum Beta- Lactamase (ESBL)-producing *E. coli* and Extended-Spectrum Beta-Lactamase (ESBL)-producing *K. pneumoniae*) [180].

The peak 26 was tentatively identified as galloylquinic acid derivative with precursor ion $[M-H]^-$ at m/z at 565 and products ions at 299, 343 and 169 [184]. The galloylquinic acid derivatives from *Copaifera langsdorffii* leaves present an antiulcer effect by decreasing the lesion size and improving the cure rate [185]. Also, a derivative of galloylquinic acid, identified as 3,5-O-di-galloylquinic acid, isolated from *Myrtus communis* leaves had an antigenotoxic effect in the K562 cell line through modulation of the expression of some DNA repair proteins, but also by modulation of some involved in the antioxidative system [186]. The peak 28 correspond to galloylquinic acid derivative with precursor ion $[M-H]^-$ at m/z at 575 and products ions at 343, 191 and 169 [183]

The peak 27 correspond to vitexin derivative , with precursor ion $[M-H]^-$ at m/z at 575 and products ions at 431, 311, 161 and 215 [187]. Vitexin, also known as apigenin-8-c-glucoside, is a c-glycosylated flavone presented in numerous medicinal plants such as wheat leaves, mimosa, bamboo, mung pea, pigeon bean [188]. Vitexin could be used in the treatment of hyperactive gut disorders because of the antispasmodic activity through activation of K_{ATP} channel [189]. Vitexin may also help to mitigate hypoxia-schemia damage by decreasing of infarct volume and reducing brain edema [190].

The peak 29 was tentatively identified as pinobanksin-5-methyl ether-3-o-acetate with precursor ion $[M-H]^-$ at m/z 327 and products ions at 285, 267, 239, 195 and 180. The product ion peaks at m/z 285 which result of the removal of $[M-H-CH_3CHO]^-$, at m/z 267 that result of the removal of $[M-H-CH_3COOH]^-$ and at m/z 239 that results of the removal of $[M-H-CH_3COOH-CO]^-$ [191] This compound found in *Coriandrum sativum* inhibited the activity of the angiotensin-converting enzyme, resulting in antihypertensive effects [191]. Also, pinobanksin-5-methyl ether-3-o-acetate, a compound present in propolis, showed antibacterial activity against *Penicillium notatum* [192].

The peak 30 corresponds to 3,5-diferuoylquinic acid and exhibited a molecular ion $[M-H]^-$ at m/z 543 and products ions at m/z 261, 191, 349 and 367. The product ions at m/z 367 correspond to a loss of ($[M-H-$ferulic acid$]^-$), m/z 349 correspond to a loss of ($[M-H-$ferulic acid$-H_2O]^-$) and m/z 191 correspond to a loss of ($[M-H-2$ferulic acid$]^-$) , which is characteristic of diferuoylquinic acids [193] This compound was previously described in other plants such as *Artemisia annua* and in grapefuit [193], [194].

Sea fingers (*Carpobrotus edulis*)

In sea fingers, 15 phenolic compounds and 5 organic acids (cinnamic aid, malic acid, citric acid, quinic acid, and succinic acid) were identified. From these 15 phenolic acids, four of them were as flavonoids (epigallocatechin, two isorhamnetin-glucoside derivative and luteolin derivative), ten of them were identified as phenolic acids (p-coumaric acid, ferulic acid-glucoside, caffeic acid derivative, four p-coumaric acid derivative, malonyl-3,4-O-caffeoylquinic acid and two ferulic acid derivatives) and one was classified as coumarin (coumarin glycoside esther), **Table 3.3**.

The peak 1 corresponds to cinnamic acid with precursor ions $[M-H]^-$ at m/z 147 and product ions at m/z 120, 119, 103 and 62 [110]. The peak 2 was tentatively identified as malic acid with precursor ions $[M-H]^-$ at m/z 133 and product ions at m/z 115, 113, 71 and 89 [111], [112]. The peak 3 corresponds to citric acid with precursor ion $[M-H]-$ at m/z 191 and fragment ions at m/z 111, 87, 85, 129 and 173. The fragment ions m/z 173, 129 and 111 results from $[M-H-H_2O]^-$, $[M-H-2H_2O-CO_2]^-$ and $[M-H-H_2O-CO_2]^-$ loss, respectively [195]. The peak 4 was tentatively identified as quinic acid with precursor ions $[M-H]^-$ at m/z 147 and product ions at m/z 120, 119, 103 and 62 [112], [114]. The peak 5 corresponds to succinic acid with precursor ions $[M-H]^-$ at m/z 117 and product ions at m/z 99 and 73 [113].

Table 3.2: Identification and quantification of phenolic compounds in Slenderleaf Ice Plant (*Mesembryanthemum nodiflorum*) **using mass spectrometry. *** concentration expressed in µg/g FW; The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalent

Peak	R_T (min)	λmax (nm)	[M-H]- m/z	HPLC–DAD–ESI-MS/MS m/z (% base peak)	Tentative Identification	Refe-rences	Concentration, average ±SD*
1	7.48	276	147	129(10); 103(10); 87(10); 85(10)	citramalic acid	[196]	
2	8.40	255	133	115(80); 89(10); 71(20)	malic acid	[111], [112]	
3	9.30	262	191	87(100); 111(80); 85(50)	quinic acid	[113], [114]	
4	9.55	252	147	103(10); 62 (100)	cinnamic acid	[110]	
5	12.08	257	117	99(10) ; 73 (10);	succinic acid	[112]	
6	26.45	290; 320	323	255 (40); 213(10); 211(10); 237(10)	pinocembrin derivative	[137]	0.84 ±0.00
7	29.72	279	553	355(50); 193(50); 155(80); 134(20); 178(10)	ferulic acid- glucoside	[116]	0.63 ±0.00
8	33.95	283; 330	305	97(80); 208(10); 225(10)	gallocatechin	[153]	3.03 ±0.00
9	34.65	272	325	163(40); 119(10)	p-coumaric acid-O-glucoside	[116]	0.48 ±0.01
10	36.75	284	305	97(100); 225(10); 208(10)	epigallocatechin	[115], [154], [155]	0.51 ±0.00
11	37.67	283	355	193(100); 178(40); 149(50); 134(50)	ferulic acid- glucoside	[116]	3.91 ±0.02
12	38.95	279; 330	321	193(15); 119(25)	ferulic acid derrivative	[132]	0.41 ±0.00
13	40.30	268; 340; 447	433	271(40); 301(20); 151(20)	avicularin	[158], [159]	2.42 ±0.00
14	41.12	279; 325	387	255(80); 211(20); 213(10); 151(40)	pinocembrin derivative	[197]	0.34 ±0.06
15	41.80	280; 330	423	197(80); 182(40); 167(45); 152(45); 125(45)	syringic acid derivative	[162]	0.18 ±0.00
16	43.57	284; 330	391	337(90); 163(10)	p-coumaric acid derivative	[126]	0.69 ±0.00

Table 3.2: I**dentification and quantification of phenolic compounds in Slenderleaf Ice Plant (***Mesembryanthemum nodiflorum*) **using mass spectrometry.*** concentration expressed in µg/g FW; The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalent **(cont.)**

Peak	R_T (min)	λmax (nm)	[M-H]- m/z	HPLC–DAD–ESI-MS/MS m/z (% base peak)	Tentative Identification	Refe-rences	Concentration, average ±SD*
17	44.32	280; 320	547	487(100); 529(10); 457(10); 427(10); 367(15); 337(15)	chrysin-6-C-ara-8-C-glu	[125]	0.64 ±0.01
18	45.82	276; 330	449	287(100)	erydictiol-O-hexoside	[165]	2.61 ±0.01
19	46.20	279; 315	287	135(50); 151(20); 107(10)	erydictiol	[167]	8.57 ±0.10
20	46.78	279; 332	307	289(60); 245(20); 179(20); 205(10)	epicatechin derivative	[154], [198]	1.06 ±0.01
21	48.67	280; 320	641	495(10); 191(10)	digalloyl quinic acid derivative	[169]	0.25 ±0.00
22	49.57	280; 352	565	301(10); 300(20); 179(30); 151(10)	quercetin dipentoside	[170]	0.13 ±0.00
23	50.30	279; 352	415	301(40); 300(10)	quercetin derivative	[172]	0.32 ±0.00
24	51.82	280; 330	481	301(10); 275(10)	hexahydroxydiphenoyl-Glucose	[177], [178]	0.18 ±0.01
25	52.93	290	565	353(70); 179(10); 111(20); 191(10)	caffeoylquinic acid derivative	[180]	0.39 ±0.00
26	53.85	279; 350	565	299(30); 343(10); 169(15)	galloylquinic acid derivative	[184]	0.16 ±0.00
27	55.40	279; 325	575	431(20); 311(10); 161(10); 215(10)	vitexin derivative	[199]	0.16 ±0.00
28	61.37	280	575	343(30); 191(50); 169(20)	galloylquinic acid derivative	[184]	0.38 ±0.00
29	62.87	280; 320	327	285(40); 267(10); 239(10); 195(40); 180(40)	pinobanksin-5-methyl ether-3-O-ace-tate	[197]	1.55 ±0.00
30	64.28	279; 325	543	261(10); 191(10); 349(10); 367(10)	3,5-diferuoylquinic acid	[193]	0.17 ±0.00

The peak 6 was tentatively identified as p-coumaric acid with precursor ions [M−H]− at m/z 163 and product ions at m/z 119 [126]. The peak 11 corresponds to p-coumaric acid derivative with precursor ions [M−H]− at m/z 289 and product ions at m/z 163 and 119 [126]. The peak 18 was tentatively identified as a p-coumaric acid derivative with precursor ions [M−H]− at m/z 525 and product ions at m/z 119 and 163 [126]. The peak 20 was also tentatively identified as a p-coumaric acid derivative with precursor ions $[M-H]^-$ at m/z 539 and product ions at m/z 419 and 163 [126].

The peak 7 corresponds to epigallocatechin having a precursor ions $[M-H]^-$ at m/z 305 and product ions at m/z 261, 179, 221 and 219 [200].

The peak 8 was tentatively identified as ferulic acid glucoside with precursor ions [M−H]− at m/z 355 and product ions at m/z 193, 175, 160, 134 and 119 [132]. The peak 17 corresponds to ferulic acid derivative with precursor ions $[M-H]^-$ at m/z 555 and product ions at m/z 193 and 134 [120].

The peak 19 correspond to the ferulic acid derivative with precursor ions $[M-H]^-$ at m/z 757 and product ions at m/z 555, 193 and 134 [120].

The peak 9 corresponds to caffeic acid derivative with precursor ions $[M-H]^-$ at m/z 355 and product ions at m/z 179 and 134 [201]. Caffeic acid derivatives are among the main phenolic compounds in white wine [201]. A caffeic acid derivative compound, caffeic acid phenethyl ester, had an neuroprotective effect by reducing the pro-inflammatory factors expression in Alzheimer's disease model [202].

The peak 10 was characterized as coumarin glycoside ester showing a precursor ion $[M-H]^-$ at m/z 351 and product ions at m/z 145 and 307 [203]. This compound is one of the compounds found in extracts of fruits of *Firmiana simplex*, that demonstrates to have an antigenotoxic effect in Hep-G2 (human liver cancer line) [203]

The peak 12 was tentatively identified as an isorhamnetin-rutinoside derivative with precursor ion $[M-H]^-$ at m/z 767 and product ions at m/z 623 and 315. The product ion m/z 315 $[M-H]^-$ result of the loss of a fragment of m/z 308, which corresponds to a rhamnoglucoside [204]. This compound was previously identified in the peach fruit extracts and proved to have anti-neurotoxicity effect against beta amyloid proteins through reduction of ROS levels [204]. It is also one of the major compound found in cultivars of Valencia and Runner peanut [205].

The peak 13 correspond to luteolin derivative with precursor ions $[M-H]^-$ at m/z 393 and product ions at m/z 299, 255, 277 and 285 [206]. Luteolin has anticancer activity, by inhibiting the cell proliferation and inducing apoptosis [207]. The luteolin also presented antidiabetic effect, by reducing the expression of factors involved in the synthesis of lipids [208].

The peak 14 was tentatively identified as isorhamnetin-glucoside derivative with precursor ion $[M-H]^-$ at m/z 621 and fragment ions at 477, 315, 519 and 559 [129]. Isorhamnetin-3-glucoside has a protective effect against the appearance of selenite cataract, by reducing lipid peroxidation, preventing oxidative damage and preserving the function of Ca^{2+}-ATPase channel [209].

The peak 15 corresponds to malonyl-3,4-O-dicaffeoulquinic acid derivative with precursor ions $[M-H]^-$ at m/z 799 and product ions at m/z 601, 191, 515, 173 and 179 [120].

The peak 16 was tentatively identified as a p-coumaric acid derivative with precursor ions $[M-H]^-$ at m/z 525 and product ions at m/z 119 and 163 [126].

Table 3.3: Identification and quantification of phenolic compounds in Sea Fingers (*Carpobrutus edulis)* **using mass spectrometry. *** concentration expressed in µg/g FW; The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalent

Peak	R_T (min)	λmax (nm)	[M-H]- m/z	HPLC–DAD–ESI-MS/MS m/z (% base peak)	Tentative Identification	References	Concentration, average ±SD*
1	7.38	305	147	120 (10); 119(10); 103(10); 62(50)	cinnamic acid	[110]	
2	8.38	255	133	115(10); 113(10); 71(10); 89(10)	malic acid	[111]–[113]	
3	9.35	255	191	111(100); 87(60); 85(40); 129(30); 173(10)	citric acid	[112], [195]	
4	9.68	262	191	111(10); 87(10); 85(10)	quinic acid	[112], [114]	
5	12.07	262	117	99(10); 73(10);	succinic acid	[112], [114]	
6	34.55	313	163	119(10)	p - coumaric acid	[126]	10.46 ±1.67
7	36.35	307	305	261(30); 179(15); 221(10); 219(10)	epigallocatechin	[115], [200]	0.86 ± 0.11
8	38.25	328	355	193(10); 175(100); 160(40); 134(10); 119(10)	ferulic acid glucoside	[112]	0.21 ± 0.06
9	38.95	268; 325	355	179(30); 134(15)	caffeic acid glucuronide	[201]	0.30 ± 0.01
10	40.32	273; 325	351	351(40); 145(10); 307(10)	coumarin glycoside ester	[203]	1.46 ± 0.21
11	46.15	280; 311	289	163(10); 119(10)	p coumaric acid derivative	[126]	0.99 ± 0.01
12	47.78	260; 352	767	623(100); 315(20)	isorhamnetin-rutinoside derivative	[112], [204]	13.83 ± 0.10
13	48.82	260; 351	393	299(40); 255(25); 277(20); 285(10)	luteolin derivative	[206]	1.68 ± 0.10
14	50.35	260; 352	621	477(100); 315(90); 519 (88); 559(30)	isorhamnetin-glucoside derivative	[112], [129]	7.28 ± 0.10
15	54.02	328	799	601(80); 191(20); 515(20); 173(15); 179(10)	malonyl-3,4-O-dicaffeoylquinic acid	[120]	0.81 ± 0.13
16	56.62	317	525	119(10); 163(100)	p-coumaric acid derivative	[126]	7.75 ±0.24

3.1.2 Comparison of phenolic content present in the 7 halophyte plants in study

After the identification of the phenolic compounds present in the seven halophyte plants, all extracts were analyzed by HPLC-DAD to quantify their main bioactive compounds. For this purpose, four standards were used, namely gallic acid, quercetin-3-glucoside, chlorogenic acid, and quercetin-3-6-acetylglucoside. The quercetin-3-glucose was used to quantify quercetin-3-glucose and quercetin glycosylated derivatives. Chlorogenic acid was used for quantification of the chlorogenic acid and corresponding derivatives. Other flavonoids were quantified as quercetin-3-O-(6-acetylglucoside) equivalents. Other phenolic acids were quantified as a gallic acid equivalents [43]. In **Appendix 1 (Figures A.1-to A.7)**, the phenolic composition of each halophyte was detailed:

- In *S. ramosissima,* the main phenolic compounds were caffeoylquinic acid and corresponding derivatives, constituting about 78% of the total plant, **Figure A.1;**
- In *S. fruticosa*, there were several compounds with high concentration such as rhamnetin hexosyl pentoside (23.63%) caffeoylquinic acid and derivatives (17.52%), isorhamnetin 3-O-robinobioside (18.01%) and p-coumaric acid and derivatives (15,89%), **Figure A.2;**
- In *I. chritmoides*, p-coumaric acid and corresponding derivatives, and pinobanksin-5-methyl ether-3-O-acetate were the compounds at higher percentage (with 31.46% and 19.66 % respectively), **Figure A.3;**
- In *C. maritimum,* the two compounds in highest percentage were caffeoylquinic acid and p-coumaroylquinic acid (54.57% and 23.93% respectively), **Figure A.4;**
- In *M. crystallinum*, the compounds with the highest percentage are caffeoylsinapylquinic acid (34.39%) and p-coumaric acid and their derivatives (29.92%), **Figure A.5;**
- In *M. nodiflorum* , the most prominent compound was eriodictyol derivative (37.24%), **Figure A.6;**
- In *C. edulis,* p-coumaric acid and isorhamnetin derivative were the two compounds detected at higher percentage (with 36.76% and 37.27%, respectively), **Figure A.7.**

In **Figure 3.1** the total phenolic content of all halophyte plants, calculated as the sum of the main compounds identified in the HPLC analysis is presented. From the results obtained it can be concluded that *C. maritimum* presented the highest concentration of phenolic compounds (222.75 µg/g FW), followed by *S. ramosissima* (118.54 µg/g FW) and *S. fruticosa* (112.07 µg/g FW). Among these plants, *C. maritimum* distinguished for showing the highest concentration of phenolic acids (193.38 µg/g FW) whereas *S. fruticosa* showed the highest amount of flavonoids (73.51 µg/g FW). It is important to mention that *S.ramosissima* also presented high concentration of phenolic acids (112.07 µg/g FW) in contrast to the lower flavonoid content (6.47 µg/g FW). The other plants, namely *M. crystanillum, M. nodiflorum, C. edulis* and *I. chritmoides* showed lower total phenolic content (values below 60 µg/g FW).

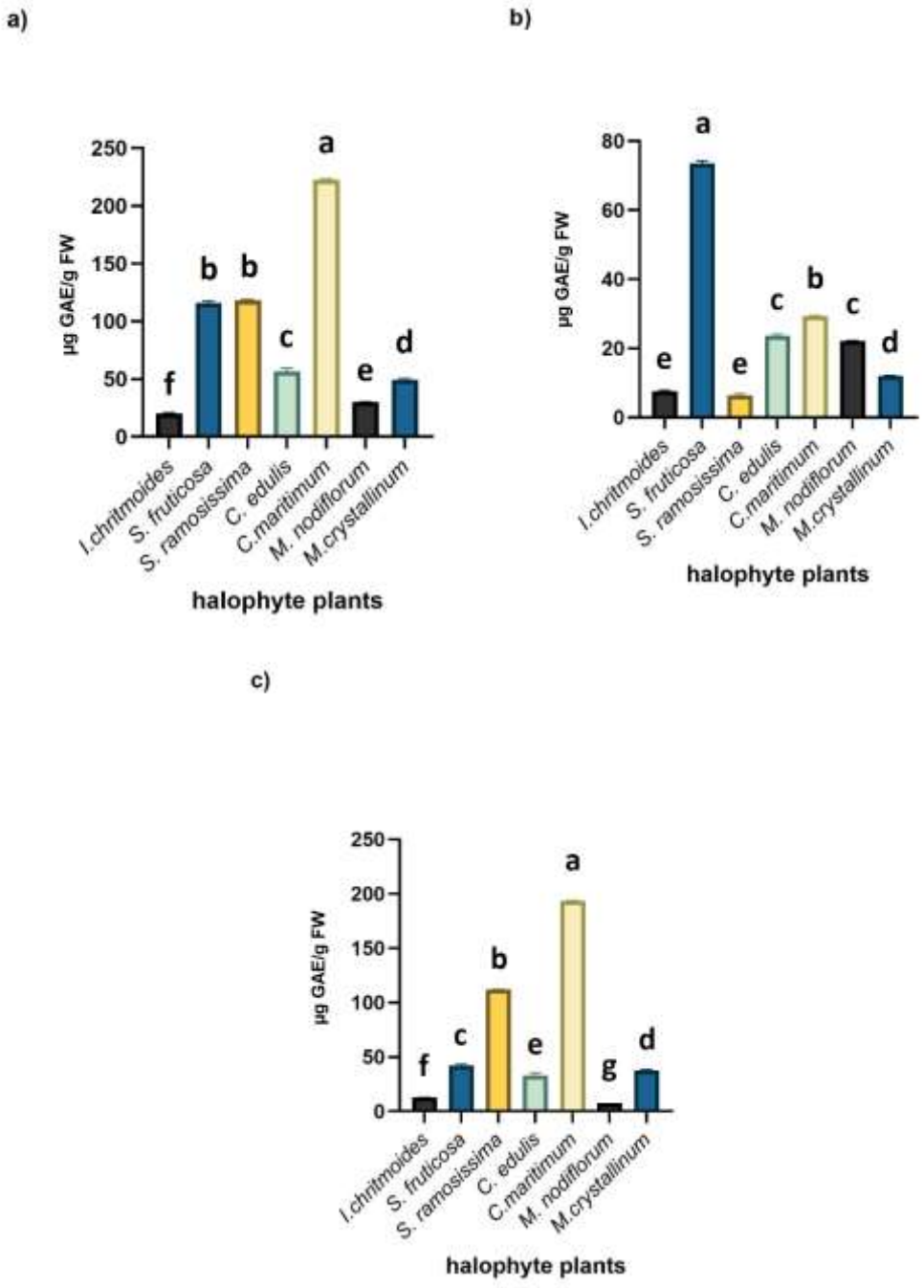

Figure 3.1: Total phenolic content of halophyte plants by chromatographic method. a) total phenolic compounds; b) total flavonoids; c) total phenolic acids; All the results are presented as µg GAE/g FW of plant. The lowercase letters (a to g) denotes significant differences according to Tukey's test ($p < 0,05$). Each bar represents average ± standard deviation (n=2)

The results of quantification by HPLC-DAD were compared with previous data obtained by the host lab using the Folin Ciocalteu assay, **Appendix 2 Figure A.8.** This is a colorimetric approach that

uses electron transfer reactions between phenolic compounds and Folin-Ciocalteu's reagent, being a simple, fast and repeatable method. This assay is the most used method to calculate the content of total phenolic compounds in food matrices and plant-based extracts [210], [209]. However this method, is also well recognized for overestimating the phenolic compounds content when compared to the sum of the individual components identified and quantified by HPLC-MS/MS or the sum of total HPLC - DAD peak area [211], [212]. In fact, TPC results obtained by Folin Ciocalteu method were 410 µg/g FW for *S. ramosissima,* 330 µg/g FW for *S.fruticosa*, 250 µg/g FW for *I. chritmoides* and *C. maritimum.* The other plants, namely *M. crystanillum, M. nodiflorum* and *I .chritmoides* showed lower total phenolic content (values bellow 120 µg/g FW). These values were higher than the ones obtained in the quantification by HPLC. All the plants, with exception of *C. maritimum*, showed values at least twice of the quantified values by HPLC. These differences could be explained by the fact that *C. maritimum* has major phenolic acids that were possibly quantified in this LC-DAD method, while the others plant presented minor compounds that could not be quantified due to the detection limit of the equipment.

The total phenolic content for the plants in study are in accordance with the ones reported in the literature. He *et al.* (2022) compared the phenolic composition of *M. crystallinum* grown under different salinization conditions. He *et al.* use a methanol: water (80; 20, v/v) in a proportion of 1 g of fresh plant for 10 mL of solvent for the phenolic's extraction of the plants. The authors obtained values of TPC ranging from 100 – 200 µg GAE/g FW . These values obtained by He *et al.* (2022) are are higher than the reported values described herein by Folin-Ciocalteu's method. (value of 100 µg GAE/g FW) [213].

Jallali *et al.* (2014) studied the phytochemical composition of *C. maritimum* and *I. chritmoides*, harvest in Tunisia salt marsh, and reporting values of TPC, by Folin-Cioalteau method, of 4.1 – 7.9 mg GAE/g DW for *C. maritimum* and values of 6.7 – 14.1 mg GAE/g DW for *I. chritmoides*, higher than the values obtain in these work, which displayed 3.04 and 2.23 mg GAE/g DW for *I.chritmoides* and *C.maritimum*, respectively. Differences in the extraction solvent and plant:solvent extraction proportion could contribute to explain differences between the results (although in Jallali *et al.* (2014) study, 80% of acetone was applied to extract the dried plants in a proportion of 1g of plant: 10 mL of solvent, in the present study EtOH 80 % was applied in a proportion of 1:50). [214], [211].

Merchaoui *et al.* (2019) compared the phenolic composition of 30 wild halophytes plants of Tunisia, *C. edulis* and *C. maritimum* were among the plants under study by Merchaoui *et al.* (2019). The higher values of 172.50 mg GAE/ g DW and 22.70 mg GAE/ g DW for *Carpobrotus edulis* and *Chritmum maritimum.* For the extraction of the phenolics, an ethanol : water (70:30, v/v) solution was used and extraction was made in a proportion of 1 g per 10 mL of the solvent. These values are much higher than obtained in this work (the values of *Carpobrotus edulis* and *Chritmum maritimum* of this work are 2.89 and 2.23 mg GAE / g DW, respectively) [215]. These values could be explained by the use of different extraction solvents and proportion, 70% EtOH against the 80% EtOH used herein [210].

As said before, the difference verified between the results of this study and the results reported in the literature , may be due to several factors relation to the extraction method, including: the type of solvent used, the ratio between solvent and sample, the composition of the solvent, among others [216].

In fact, Jallali *et al.* (2014), Merchaoui *et al*. (2019) and He *et al. (*2022) used different extraction solvents (80 % MeOH, 80 % acetone and 70% respectively), than the one employed in this work (80 % ethanol) [213]–[215]. Besides, these differences between the values reported in the literature and the results of this work for TPC, can also be explained by variations in some environmental factors related to the conditions of plant growth, such as saline stress, UV radiation, among other environmental changes [84]. Because the plants used in this study were grown under aquaponic conditions, they were insulated from some of the harsh environmental conditions and abiotic stressors that wild plants face, which contributed for a reduction in secondary metabolite levels related to the antioxidant system, such as phenolic compounds [21], [213]. Other variables, such as genetic differences and the degree of the maturation of the plant could also have impact in the phenolic composition of the plant [217].

Overall, taking into account the total phenolic content and the phenolic composition of the plants, for *in vitro* digestion studies, two samples were selected:

i. Based on the highest total phenolic content and highest concentration of phenolic acids (**Appendix 2)**, as well as high productivity and acceptability/ demand in the European Market, *S. ramosissima* was one of the selected plants [70];
ii. Based on the highest total flavonoids content and great variability in the phenolic composition (**Appendix 1**), *S. fruticosa* was also a selected plant.

3.2 Impact of the *in vitro* digestion on the phenolic content and antioxidant activity of halophyte plants

3.2.1 Phytochemical characterization and antioxidant capacity of raw material

S. fruticosa and *S. ramosissima* were lyophilized and subjected to a conventional extraction process with 80% ethanol and ultrasonic sonication to increase the extraction efficiency of phenolic compounds and antioxidant compounds. **Table 3.4** show the TPC, HOSC, and ORAC values of these two plants.

Table 3.4: TPC (expressed as mg GAE/g DW) HOSC and ORAC (expressed as µmol TEAC/g DW) of *Salicornia ramosissima and Sarcocornia fruticosa* (a-b indicates values statistically different, t-test for independent samples, $p<0.05$)

Species	TPC (mg GAE/g DW)	HOSC (µmol TEAC/g DW)	ORAC (µmol TEAC/g DW)
Salicornia ramosissima	7.87 ± 0.94^{a}	134.86 ± 23.78^{a}	241.93 ± 41.31^{a}
Sarcocornia fruticosa	4.60 ± 0.31^{b}	112.07 ± 21.88^{a}	151.07 ± 21.99^{b}

Salicornia ramosissima (7.87 mg of GAE/g DW) has a higher TPC value than *Sarcocornia fruticosa* (4.60 mg of GAE/g DW), **Table 3.4**. Sánchez- Gávilan *et al.* (2021) compared the bioactive compounds of different populations of *Salicornia patula*, from Spain. The authors obtained TPC values

ranging from 2.99 mg GAE/g DW to 4.21 mg GAE/g DW, with explained variation attributed to the different collection places [218]. Grigore *et al.* (2015) studied the *Salicornia europaea* plant collected in salt areas of Romania, reporting values of 1.04 mg GAE/ g DW [219]. Oliveira – Alves *et al.* (2021) obtained TPC values of 9.74 mg GAE/g DW for *S. ramosissima* grown using traditional farming in 'Ria de Aveiro' and dried using the same method as described herein [57].

Regarding *Sarcocornia*, Sánchez-Gavilán *et al.* (2021) described the bioactive compounds, including phenolic compounds present in methanolic extracts of three plant species belonging to the genus *Sarcocornia* (*S. perennis, S. pruinosa and S. alpini*) [84]. In the Folin-Ciocalteau's assay, the authors obtained values for the total phenolic compounds of the plants belonging to the genus *Sarcocornia* between 3.231 mg GAE/g plant DW and 3.892 mg GAE /g plant DW [84]. Antunes *et al*. (2021) reported TPC values of 3.38 and 3.56 mg GAE/ g DW for *Sarcocornia perennis* produced by traditional farming [70].

The antioxidant activity of *S. ramosissima* and *S. fruticosa* were measured by the ORAC and HOSC assays, **Table 3.4.** The *S. ramosissima* has a higher value of ORAC assay than *S.fruticosa*, with both plants having a similar value of HOSC assay. *S. ramosissima* showed higher values of 134.86 and 241.93 µmol TEAC/g DW for the HOSC and ORAC assay, respectively, compared to *S. fruticosa*, which presented values of 112.07 and 151.07 µmol TEAC/g DW for the HOSC and ORAC assay, respectively. Antunes *et al.* (2021) obtained values of ORAC ranging from 32.3 and 90.6 µM Trolox/g of DW for *Sarcocornia perennis* grown naturally in 'Ria Formosa' [70]. Alves *et al.* (2021) obtained values of 237.20 and 418.81 µmol TEAC/g DW for the HOSC and ORAC assays, respectively, being these values referring to extracts of *S.ramosissima* [57] .

The differences between the values reported in the literature for TPC, HOSC and ORAC could be attributed to the different growing conditions, biological factors (such as population and individual variation) and/or extraction method employed [21] [220],[217].

3.2.2 Bioaccessibility of bioactive compounds from *Salicornia ramosissima* and *Sarcocornia fruticosa* : total phenolic content and antioxidant capacity of digestive fractions

Dried *S.ramosissima* and *S. fruticosa* were submitted to an *in vitro* digestion process (upper gastrointestinal tract) using the standardized INFOGEST protocol [97]. For both plants, the oral, gastric and intestinal phases were collected and analyzed for the total phenolic content, **Figure 3.2.**

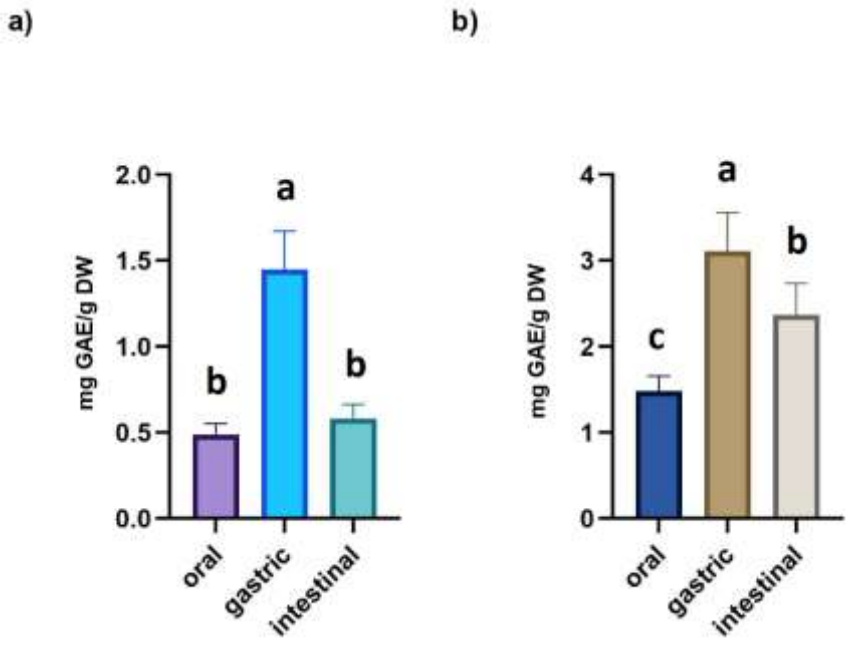

Figure 3.2: Total phenolic content (TPC) values obtained for *Salicornia ramosissima* and *Sarcocornia fruticosa* along the different phases of *in vitro* digestion. *a) S. ramosissima ; b) S. fruticosa.* All results are expressed in mg GAE/g DW. Each bar represents average ± standard deviation (n=2). The lowercase letters (a-c) denotes the significant differences between the digestion phases, Tukey's test, $p<0.05$.

Concerning *S. ramosissima*, after the oral phase - only 6.2% of the total phenolics were bioacessible - 0.49 mg GAE/g DW were liberated from the food matrix. In the gastric phase, an increase on the phenolic content was registered, reaching a value of 1.45 mg GAE/g DW, with an improvement of the bioacessibility to 18.4%. Finally, when passing to the intestine, a decrease in TPC was observed to 0.58 mg GAE/g DW, which corresponds to a bioacessibility of 7.4%. A similar trend was verified for *S. fructicosa*. After the oral phase, the total bioaccessible phenolic were 1.49 mg GAE/g DW, corresponding to a bioacessibility of 32.3%. After two hours of stomach digestion, the amount of bioaccessible total polyphenols increases to 3.11 mg GAE/g DW, corresponding to a bioacessibilty of 67.5%. The amount of total bioaccessible phenolics decreased as a consequence of the pH increase, from the gastric acidic stomatch to the intestinal phase of digestion, resulting in a value of 2.4 mg GAE/g DW, which corresponds to a bioaccesibility of 51.5%, **Figure 3.2.**

Our results are in agreement with previous studies, which analyzed different matrices such as grapes, apple varieties, pomegranate, oreganos, fruit seeds, showing the same behavior of an increment in total phenolic content in the gastric phase and subsequent decrease in the intestinal phase [221]–[226]. This behavior can be explained by the fact that in solid matrices before compounds being bioaccessible and eventually bioavailable, they must be firstly extracted [227]. The first components of gastrointestinal tract (the oral and gastric cavity) acted as an "extractor", causing plant tissue to be breakdown and phenolic compounds to be released, by both mechanical (mastication) and chemical action during the oral and gastric stages [227], [228] The increase of the bioaccesibility in the gastric phase is a result of

the hydrolysis of certain phenolic compounds linked to other components of the matrix, such as phenolics linked to the cellular walls and proteins, which it's induced by the pepsin activity and acidic pH [229]. In fact, there is a large quantity of phenolic compounds that are linked to cells walls, proteins and polysaccharides by hydrophobic and hydrophilic interactions, ethers and ester bonds and hydrogen bonds [230]. Saura-Calixto *et al.* (2007) report that digestive enzyme action may release phenolic compounds attached to these high molecular weight compounds, which might explain the large rise in phenolic compounds after the gastric phase [231]. In the intestinal phase, there is a decrease in TPC for both plants. The neutral pH of the intestine (pH=7) seems to be the explanation for the decrease of TP content, as a large majority of the phenolic compounds are highly unstable at neutral or mild basic pH, being more resistant to the acidic conditions of the stomach [225], [232]–[234].

To understand how the bioactivity of *S.ramosissima* and *S.fruticosa* changed throughout the *in vitro* digestion process, the antioxidant activity of the conventional extract and all the digestive fractions was determined by HOSC and ORAC assays. The HOSC and ORAC assays showed a similar trend for *S.ramosissima* and *S.fruticosa,* **Figures 3.3 and 3.4**. In relation to HOSC and ORAC assays for the different fractions of the digestion process of *S. ramosissima*: in the oral phase obtained a value of 13.33 µmol and 22.21 µmol TEAC/g DW (corresponding to 9.9% and 9.2% of the value determined in the undigested extract, respectively) were determined; in the gastric phase a value of 124.83 and 155.72 µmol TEAC/g DW (corresponding to 92.6% and 64.7% of the determined value in the undigested extract, respectively) were obtained; in the intestinal phase a value of 79.49 and 62.96 µmol TEAC / g DW (corresponding to 58.9% and 26.0% of the determined value in the undigested extract, respectively) were determined, **Figure 3.3**.

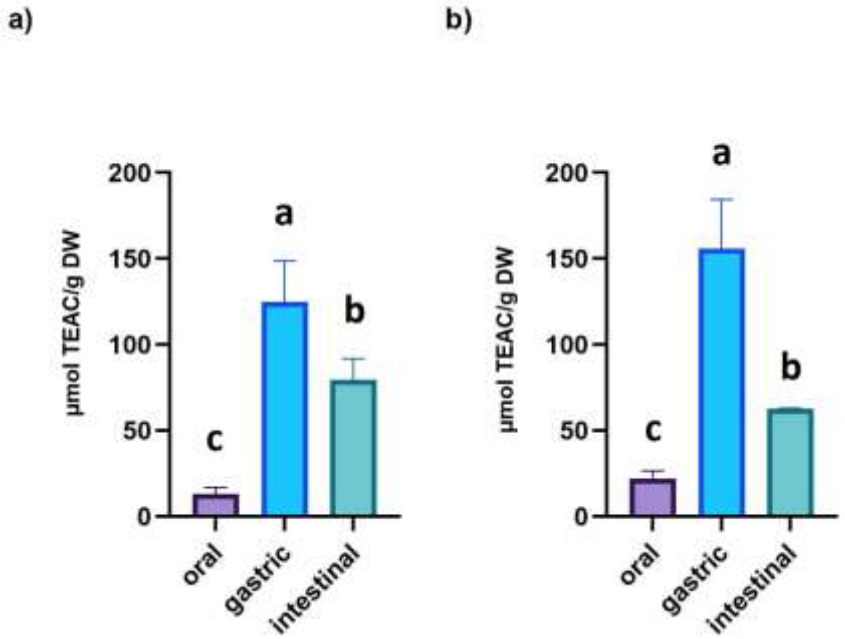

Figure 3.3: HOSC (a) and ORAC (b) values for *Salicornia Ramosissima* along the different phases of *in vitro* digestion All results are expressed in µmol TEAC/g DW. Each bar represents average ± standard deviation (n=2). The different lowercase letters indicate significant differences between the digestion phases, Tukey's test, $p<0.05$.

In relation to HOSC and ORAC assays applied for the different fractions of the digestion process of *S. fruticosa*: in the oral phase a value of 28.13 and 30.93 µmol TEAC/g DW (corresponding to 25.10% and 20.47% of the determined value in the undigested extract, respectively) were obtained; in the gastric phase a value of 69.12 and 107.76 µmol TEAC/g DW (corresponding to 61.68% and 71.33% of determined value in the undigested extract, respectively) were obtained, and in the intestinal phase values of 35.10 and 80.21 µmol TEAC/g DW (corresponding to 31.32 % and 53.09 % of the determined value in the undigested extract , respectively) were obtained, **Figure 3.4.**

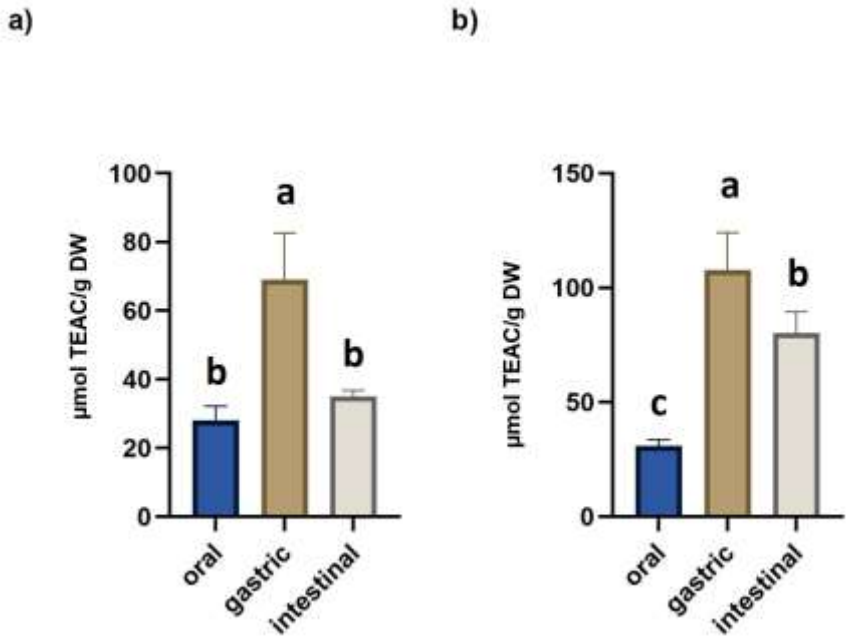

Figure 3.4: HOSC (a) and ORAC (b) values for *Sarcorconia fruticosa* along the different phases of *in vitro* digestion. Each bar represents average ± standard deviation (n=2). All results are expressed in µmol TEAC/g DW. The lowercase letters (a-c) denotes the significant differences among the phases digestive according to Tukey's test ($p < 0.05$)

The antioxidant activity after the oral digestion was much lower than the antioxidant activity of the conventional extracts, which corresponds to the lower content of total phenolics extracted after this phase. After gastric digestion, the antioxidant capacity undergoes a significant increase when compared to oral digestion, which was in agreement with the total phenolic content, which also showed an increase after the transition from one phase to another. The antioxidant capacity decreased after the intestinal phase, similarly to what happens with TPC. Structural transformations as a consequence of the racemization process during the intestinal phase justified the differences in the antioxidant activity [230]. The racemization of compounds is known to be affected by pH, resulting in two enantiomers with distinct reactivity [235]. As the pH increases so does the racemization of the compounds, which makes the

antioxidant compounds more reactive under stomach pH conditions (pH=3) than under intestinal pH conditions (pH=7) [235].

Possible associations and even correlations between the content of phenolic compounds and the antioxidant activity of digestive fractions have been established by several authors [224], [226], [232], [236]–[240]. **Table 3.5** shows the Pearson's r correlations between TPC and antioxidant values (ORAC and HOSC assays). Results showed higher correlations between TPC and ORAC or HOSC for *S. fruticosa* (Pearson's r>0.97) than for *S. ramosissima* (Pearson's r<0.9) The low correlation between TPC and HOSC in *S. ramosissima* could be explained by the presence of other compounds in digested fractions, namely peptides or aminoacids that are modified and released from the food matrix during the digestion process, that may present scavenging effect of hydroxyl radicals [122].

Table 3.5: Pearson's r correlation of TPC *vs* ORAC and TPC *vs* HOSC for *S. ramosissima* and *S. fruticosa*

	S. ramosissima		***S. fruticosa***	
	TPC *vs* ORAC	**TPC *vs* HOSC**	**TPC *vs* ORAC**	**TPC *vs* HOSC**
Pearson's r	0.8882	0.6447	0.9783	0.9859

3.2.3 Identification of phenolic compounds throughout the *in vitro* digestion process

The phenolic compounds in the conventional extracts of *S. ramosissima* and *S.fruticosa* were identified using HPLC-DAD by comparison with the previously collected data. The retention time, maximum absorption in the UV/VIS spectrum, and elution order were all checked to identify each compound, **Table 3.6** and **3.7**.

Table 3.6: Phenolic compounds identified and quantified in *Salicornia ramosissima* extract using HPLC-DAD; * compound confirmed using a commercial standard (quantification expressed as µg/g DW); ** Chlorogenic acid and derivatives were quantified as a chlorogenic acid equivalent. Flavonoids were quantified as a quercetin-3-glucose equivalent. Other phenolic acids were quantified as gallic acid equivalent.

Nº	**putative identification**	**R_T (min)**	**λMax (nm)**	**Concentration average ± SD (µg/g DW)****
1	neochlorogenic acid	31.08	300; 326	195.62 ± 0.43
2	gallocatechin	32.26	280	193.62 ± 3.39
3	chlorogenic acid *	37.30	300; 326	1793.55 ± 5.30
4	p-coumaroylquinic acid	42.86	313; 293	14.10 ± 0.05

Table 3.6: Phenolic compounds identified and quantified in *Salicornia ramosissima* extract using HPLC-DAD; * compound confirmed using a commercial standard (quantification expressed as µg/g DW); ** Chlorogenic acid and derivatives were quantified as a chlorogenic acid equivalent. Flavonoids were quantified as a quercetin-3-glucose equivalent. Other phenolic acids were quantified as gallic acid equivalent. **(cont.)**

Nº	putative identification	R_T (min)	λMax (nm)	Concentration average ± SD (µg/g DW)**
5	3,4-dicaffeoylquinic acid	48.76	300; 325	2328.13 ± 51.94
6	3,5-dicaffeoylquinic acid	49.83	300; 327	3352.19 ± 82.44
7	4,5-dicaffeoylquinic acid	50.65	296; 327	606.26 ± 12.07
8	caffeoylhydrocaffeoylquinic acid	51.16	290; 327	245.16 ± 2.13

Table 3.7: Phenolic compounds identified and quantified in *Sarcocornia fruticosa* extract using HPLC-DAD; * compound confirmed using a commercial standard (quantification expressed as µg/g DW) Chlorogenic acid and derivatives were quantified as a chlorogenic acid equivalent. Flavonoids were quantified as a quercetin-3-glucose equivalent. Other phenolic acids were quantified as gallic acid equivalent.

Nº	putative identification	R_T (min)	λMax (nm)	Concentration average ± SD (µg/g DW)
1	neochlorogenic acid	31.07	282; 324	158.43 ± 2.27
2	gallocatechin	32.26	279	178.43 ±5.11
3	chlorogenic acid*	37.30	300; 326	647.71 ± 9.68
4	eriodyctiol-O-hexoside	46.09	315	2.41 ± 0.21
5	rhamnetin hexosyl pentoside	47.10	354; 255	102.14 ± 3.25
6	isorhamnetin 3-O-robinobioside	48.27	256; 351	73.75 ± 3.93
7	3,4-dicaffeoylquinic acid	48.77	296; 325	398.72 ± 34.78
8	3,5-dicaffeoylquinic acid	49.84	296; 327	964.02 ± 156.42
9	4,5-dicaffeoylquinic acid	50.65	294; 326	317.20 ± 43.65

The presence and concentration of the compounds identified in the conventional extracts were measured in the digestive fractions to determine their bioaccessibility throughout the various stages of the process. Calibration curves from three available standards were used to quantify the phenolic components. Chlorogenic acid was used for the quantification of chlorogenic acid and its derivatives, gallic acid was used for the quantification of other phenolic acids and quercetin-3-glucoside was used to for the quantification of flavonoids.

As presented in **Table 3.6** and **Appendix 3**, neochlorogenic acid, gallocatechin, chlorogenic acid, ferulic acid-glucoside, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and caffeoyhidrocaffeoylquinic acid were identified in the extract of *S. ramosissima* by HPLC-DAD. All these compounds have already been reported as existing in the composition of *S.ramosissima* [57], [59], [60]. The predominant compounds quantified were 3,5-dicaffeoylquinic acid, 3,4 - dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and chlorogenic acid. These results were in accordance

with Oliveira-Alves *et al.* (2021) and Pinto *et al.* (2021) who found that caffeoylquinic acid derivatives are the major phenolic acids compounds identified in *Salicornia ramosissima* [57], [59].

In relation to *Sarcocornia fruticosa* the following compounds were identified by HPLC-DAD: neochlorogenic acid, gallocatechin, chlorogenic acid, erydictiol-O-hexoside, rhamnetin hexosyl pentoside , isorhamnetin 3-O-robinoside, 3.4-dicaffeoylquinic acid and 3.5-dicaffeoylquinic acid and 4.5-dicaffeoylquinic acid, **Table 3.7** and **Appendix 3**. Some of these compounds, such as chlorogenic acid and neochlorogenic acid have already been detected in plants belonging to the genus *Sarcocornia* [79], [92]. The major compounds 3,5- dicaffeoylquinic acid, 3,4 - dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and chlorogenic acid were quantified. In *S. fruticosa*, chlorogenic acid was one of the main detected compounds [79].

Regarding the behavior of compounds when subjected to gastrointestinal digestion, **Table 3.8** and **3.9** and **Appendix 3,** the caffeoylquinic acids had a low liberation in the oral phase, having caffeoylquinic acids a bioacessibility of 8.8 % and 1.3 % for *S. ramosissima* and *S. fruticosa*, respectively. The explanation for this is the brief contact of oral fluid with the bolus before reaching the stomach, having this way an impact considerably less evident in the liberation of phenolic compounds than that of the subsequent digestive phase [241]. There is an increase in the concentration of caffeoylquinic acids compounds after the gastric phase, having these compounds a bioacessibility of 33.2% and 37.8% *for S. ramosissima and S. fruticosa.* During this phase the acidic condition and the gastric enzyme action promotes the release of attached caffeoylquinic acid derivative compounds present in glycosidic forms and their bioaccesibility [231], [242]. In the intestinal phase, the caffeoylquinic acid derivatives are not detected. Several studies have monitored the caffeoylquinic acids derivatives present in different matrices when subjected to an *in vitro* digestion process. Vallejo *et al.* (2004) discovered that the composition of caffeoylquinic acid derivatives was considerably reduced by pepsin digestion of broccoli, with only 20.0% of these compounds being bioaccessible in the end of the intestinal digestion [243]. After stomach and intestinal digestion of herbal tea *Artemisia frigida,* Olennikov *et al.* (2019) found a decline in total caffeoylquinic acid content in the stomach (10.6%) and a drop in total caffeoylquinic acid content in the intestine (35.2%), compared to initial values [244]. Friedman *et al.* (2000) demonstrated that chlorogenic acid is particularly degradable between pH 7 and 11, which is similar to the intestinal pH [245]. Also, Siracusa *et al.* (2011) compared the digestion of the chlorogenic acid of two different matrices (*Crithmum maritimum* and *Capparis spinosa*) and the commercial standard [246]. They verified, for the chlorogenic acid, a completely different behavior in the commercial metabolite, the *Capparis spinosa* and the *Crithmum maritimum* digestion with losses of 95.7, 33.0 and 81.7%, respectively, at the end of the intestinal phase [246]. The *in vitro* digestion of blackberries, revealed a pattern similar to the one obtained in this work, for neochlorogenic acid, which was continuously released in the oral and gastric phase and a decreased in the gastric phase [247]. Differences between the behavior of caffeoylquinic acids described above is a result of the food matrix itself (whether the matrix is liquid or solid), and of the applied conditions in the *in vitro* simulation of the digestion process [246], [248], [249]. In fact, the comparison of food components' bioaccessibility can be impaired by the different conditions / methodologies used to mimic the digestion process [101], [249]. Siracusa *et al.* (2011) also argued that the

interaction with other minority components and even the matrix may have an influence on the bioaccesibility of individual phenolic compounds when subject to an *in vitro* digestion process [242].

In relation to gallocatechin, the concentration of this compound in gastric and intestinal fractions was higher than the one determined in the conventional (undigested) extract, resulting in high % of bioacessibility (> 100%). This behavior for compounds related to gallocatechin, such as epicatechin and catechin, has been verified in another food matrix such as cocoa and blackberries [247], [250]. It is possible that the extraction process used was not strong enough to totally break the cellular components with which these phenolic compounds are linked. Only in the circumstances of pH, temperature and enzymatic treatments present in the *in vitro* digestion process, these phenolic compounds linked with some cellular components can be adequately released [250].

Other flavonoids present in *S. fruticosa* were also more bioacessible in the digestive fraction (oral and gastric) being also detected in the intestinal phase. This conclusion is consistent with the findings of Tagliazucchi *et al.* (2010) who discovered that flavonoids, except the anthocyanins, are less degradable at pH conditions of the intestine [251].

The enzymatic precipitation process applied during sample preparation, prior HPLC analysis, appears to have failed in the intestinal fractions obtained from *S. ramosissima* and *S. fruticosa*, since there are peaks that appear in the intestinal fraction as well as in the respective control tube, indicating the presence of interfering enzymes and other SIF constituents. Besides, the phenolic compounds may chemically interact with the digestive enzymes and bile acids, contributing to reduce the phenolic compounds' content when analyzed by HPLC-DAD [252]. Some small peaks, that could be phenolic metabolites derived from the digestive process, were present in the intestinal fraction but their identification was not possible by HPLC-DAD. Therefore these compounds should be further identified by mass spectrometry.

Conventional extraction data are frequently used to determine the quantity of phenolic compounds consumed in daily human meals [225], [253]. Despite conventional extraction methods are widely used to characterize food matrices in terms of bioactive constituents, these methods do not predict the bioacessibility. In fact, as observed in this work, from the total compounds identified in the conventional extract of both halophyte plants, only few of these compounds will actually be bioaccessible after the digestion [254] Despite this, the matrix still contains phenolic compounds that were not extracted during the gastrointestinal process, but that can be released and converted by the colonic microflora into molecules with a potential positive biological effect for the cells of the large intestine [255].

Table 3.7: Bioaccessibility of phenolic compounds in *Salicornia ramosissima;* The lowercase letters (a to d) and uppercase letter (A to F) denote significant differences according toTukey's test ($p < 0.05$) (quantification of the compounds expressed in µg/g DW)

compound	*S. ramosissima* (µg/g DW)	Oral phase (µg/g DW)	bioaccessibility (%)	Gastric phase (µg/g DW)	bioacessibility (%)	Intestinal phase (µg/g DW)	bioaccessibility (%)
Caffeoylquinic acids	8520.91^{A}	752.07^{Cb}	8.8	2824.42^{Ba}	33.2	not detected	not detected
Flavanol (Gallocate-chin)	193.62^{C}	363.10^{Aa}	187.5	327.56^{Bb}	169.2	not detected	not detected

Table 3.8: Bioaccessibilty of phenolic compounds in *Sarcocornia fruticosa* The lowercase letters (a to i) and uppercase letters (A to F) denote significant differences according to Tukey's test ($p < 0.05$) ((quantification of the compounds expressed in µg/g DW)

compound	*S. fruticosa* (µg/g DW)	Oral phase (µg/g DW)	bioaccessibility (%)	Gastric phase (µg/g)	bioacessibility (%)	Intestinal phase (µg/g DW)	bioaccessibility (%)
Caffeoylquinic acids	2486.09^{A}	32.22^{Cb}	1.3	940.16^{Ba}	37.8	not detected	not detected
Flavanol (Gallocate-chin)	178.43^{B}	188.47^{Bb}	105.6	329.14^{Aa}	184.5	not detected	not detected
Other flavonoids	178.31^{C}	161.62^{Cc}	90.6	298.87^{Aa}	167.6	205.38^{Bb}	115.18

4

CONCLUSION

Although there are already many studies about the nutritional content and the content of phenolic compounds in halophyte plants, few studies have been carried out in order to understand the bioaccessibility and bioavailability of these compounds and their possible positive health effects. In this master's thesis, two halophyte plants, namely *S. ramosissima* and *S. fruticosa,* produced by hydroponics, in Portugal, were subjected to an *in vitro* digestion process in order to evaluate for the first time the bioaccessibility of phenolic compounds throughout the different stages of the digestion process.

These plants were selected as they present high phenolic content (410 µg/g FW in the colorimetric assay and 119 µg/g FW in the Chromatographic quantification for *S. ramosissima* and 330 µg/g FW in the colorimetric assay and 112 µg/g FW in the Chromatographic quantification for S. *fruticosa*) and great variability of phenolic compounds, such as rhamnetin hexosyl pentoside, caffeoylquinic acid derivatives and isorhamnetin 3-o-rubinoside, when compared to other halophyte species.

Both plants, when subjected to the *in vitro* digestion process presented a behavior in relation to the total phenolic content similar to that already verified in other food matrices, with an increase in the bioaccessibility in the gastric phase and a decrease in the intestinal phase. Antioxidant activity assays (HOSC and ORAC) for both plants also revealed a similar behavior. The results of the gastric phase are explained by the acid pH of the stomach and the action of the enzyme pepsin.

Using HPLC-DAD, it was possible to detect the presence of caffeoylquinic acid and its derivatives, galocatechin and other flavonoids (eriodyctiol-hexoside,rhamnetin hexosyl pentoside, isorhamnetin 3-O-robinobioside) in the undigested extracts and digestive fractions. From the results obtained in the HPLC-DAD, most of the phenolic compounds present in *S. ramosissima* and *S. fruticosa*, namely caffeoylquinic acid, corresponding derivatives, and gallocatechin, were not detected in the intestinal phase, suggesting the limited bioacessibility of these compounds and consequently they will not be absorbed by the epithelial cells of the intestine.

5

FUTURE PERSPECTIVES

Despite previous data indicating the richness of the halophyte *Salicornia ramosissima* and *Sarcocornia fruticosa* in phenolic compounds, this study showed that the majority of the phenolic compounds with the exception of some flavonoids in *Sarcocornia fruticosa,* were not detected during the digestive process. However, further studies should be performed using mass spectrometry analysis to investigate other phenolic compounds' metabolites present in intestinal phases and to confirm the phenolic compounds' putative identification performed herein. Also in the future, it is necessary to improve the sample treatment to allow a better identification of phenolic compounds in the intestinal phase. The optimization of extraction procedures such as ultrasound extraction combined with acidic hydrolysis could be applied in the future to improve the extraction of bound compounds and consenquently their identification.

In future it will be also important to complement this in *vitro* digestion study with assays involving colonic bacteria to have a more realistic gastrointestinal model. By using this model we could further investigate the colonic fermentation of the compounds that remained in the food matrix to evaluate their health promoting effect through the modulation of the gut microbiota. Importantly, the investigation of the bioaccessibility and bioavailability of halophyte plants should proceed by expanding the study to other plants like *Chritmum maritimum* rich in phenolic compounds such as p-coumaroylquinic acid and caffeoylquinic acid.

It would also be interesting to perform digestion of extracts instead of the plant in order to ascertain the bioaccessibility in an extract rich in phenolic compounds and its possible application in the development of nutraceutical formulations.

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APPENDICES

Appendix 1: Phenolic composition of halophyte plants

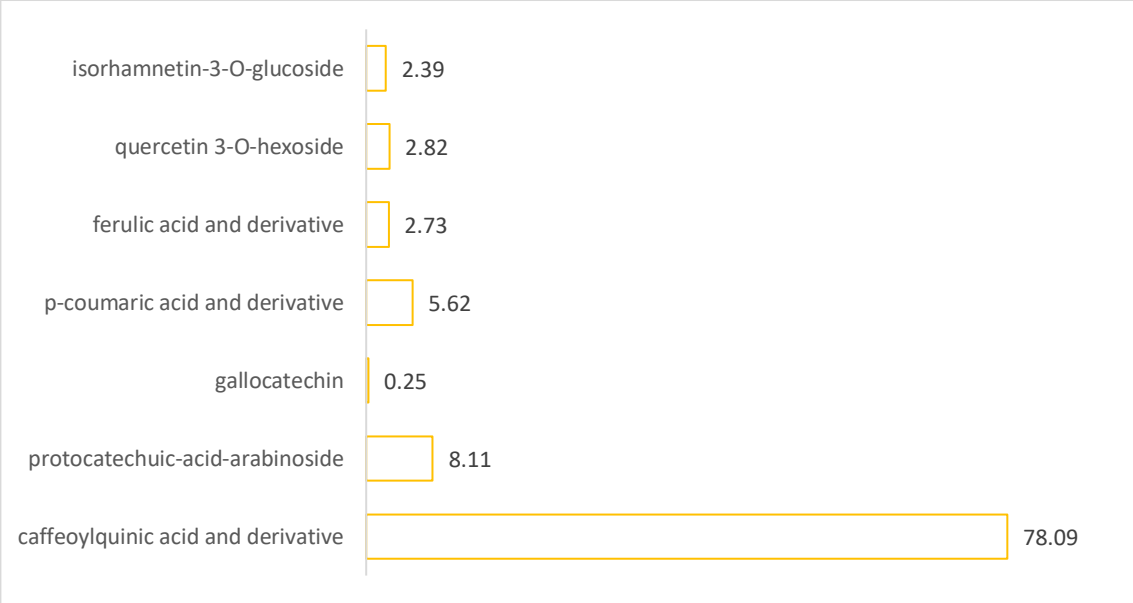

Figure A.1: Relative percentage of the phenolic compounds identified in *Salicornia ramosissima* (Salicornia)

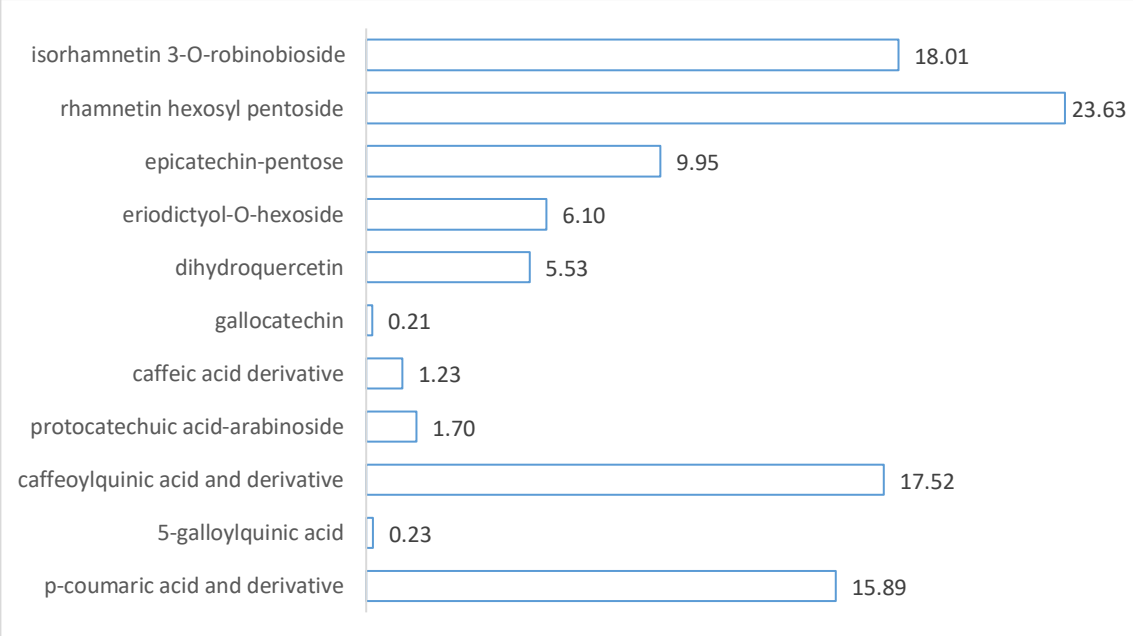

Figure A.2: Relative percentage of the phenolic compounds identified in *Sarcocornia futicosa* (Sarcocornia)

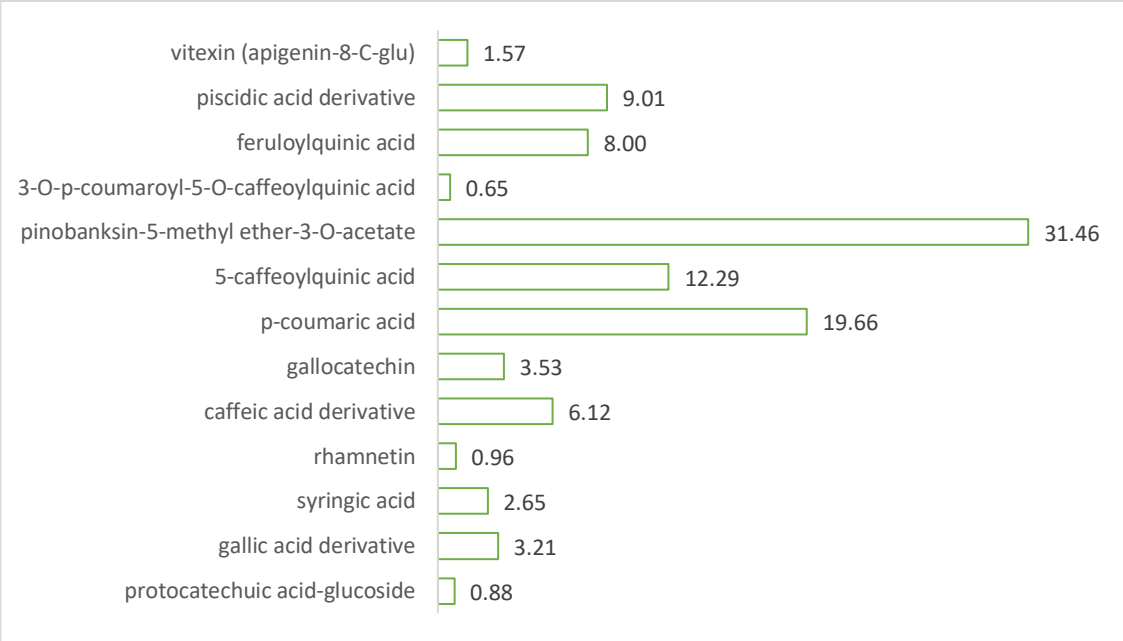

Figure A.3: Relative percentage of the phenolic compounds identified in *Inula chritmoides* (Inula)

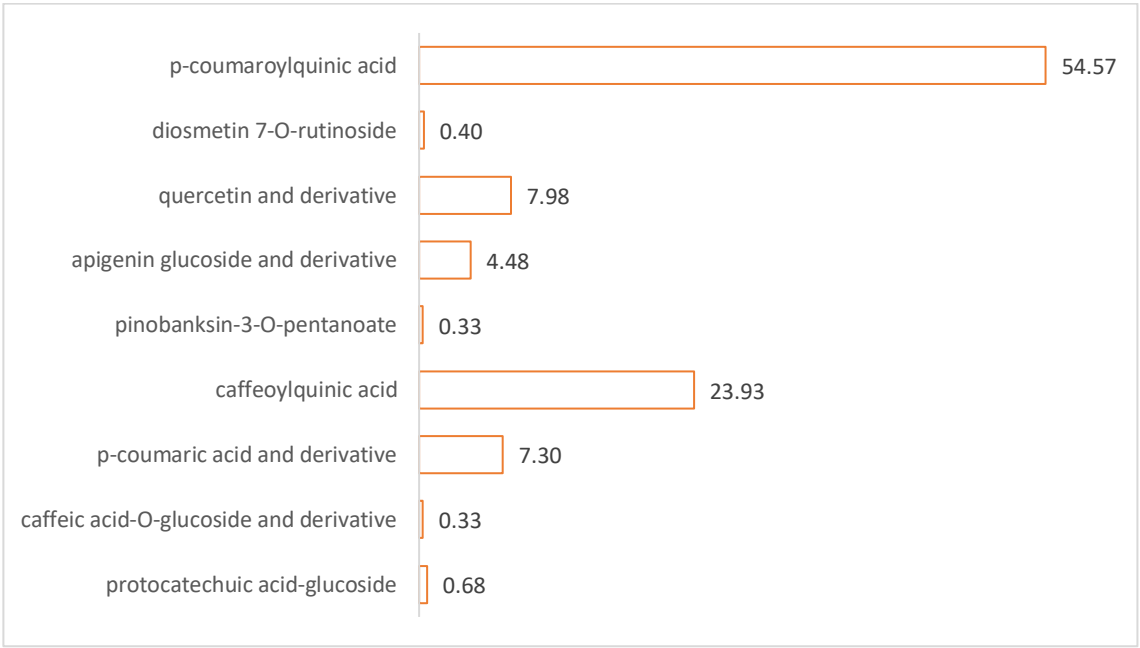

Figure A.4: Relative percentage of the phenolic compounds identified in *Chritmum maritimum* (Sea fennel)

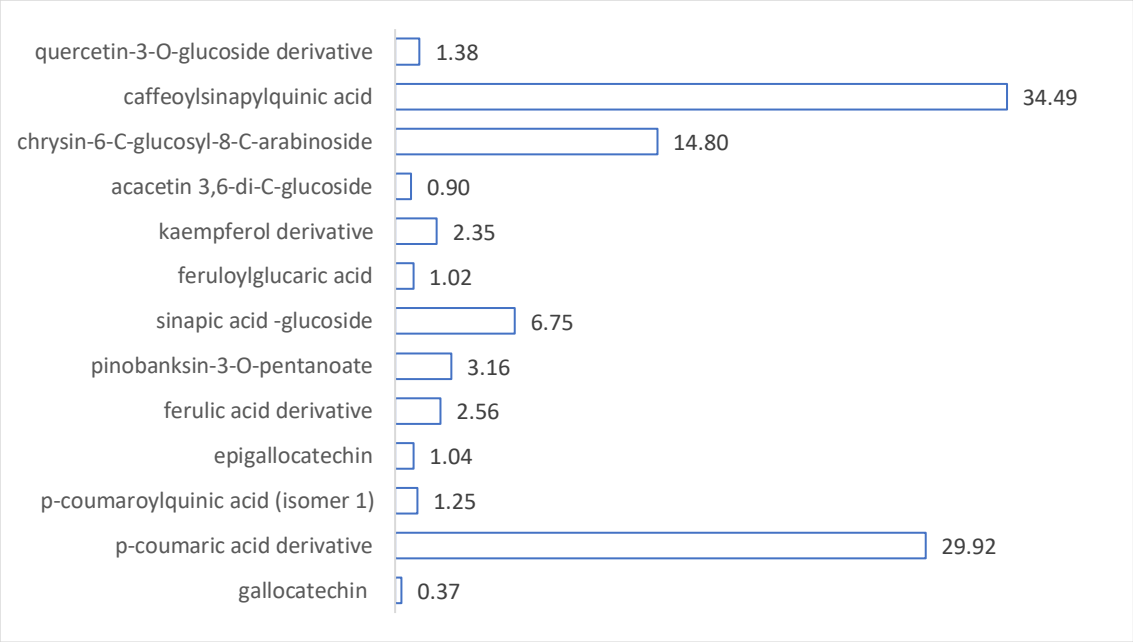

Figure A.5: Relative percentage of the phenolic compounds identified in *Mesembryanthemum crystallinum* (Ice plant)

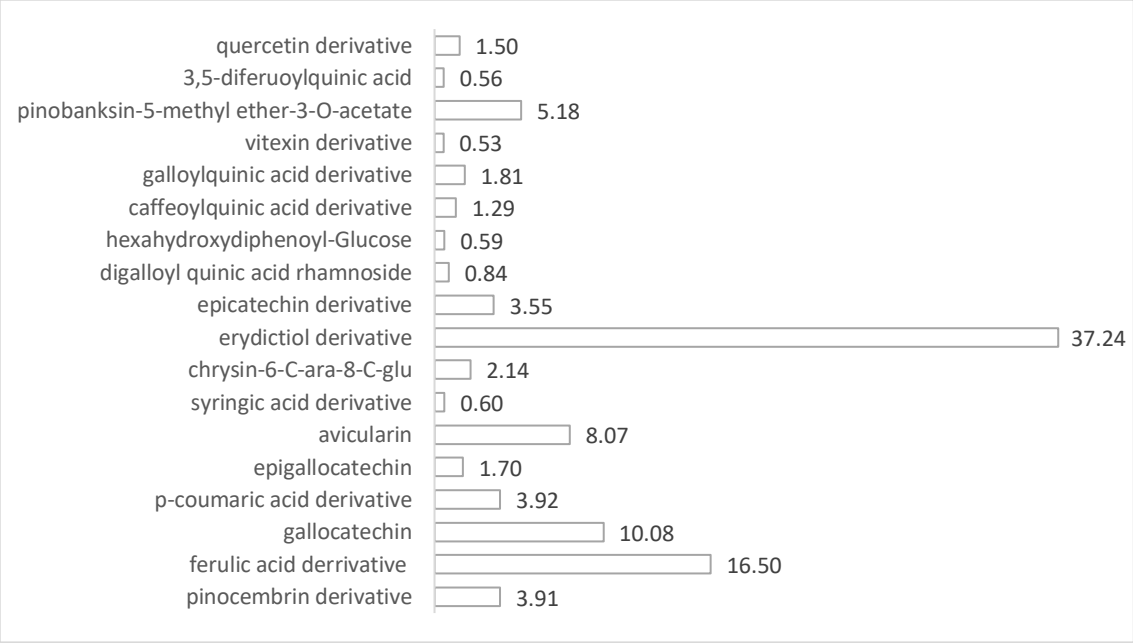

Figure A.6: Relative percentage of the phenolic compounds identified in *Mesembryanthemum nodiflorum* (Slenderleaf Ice plant)

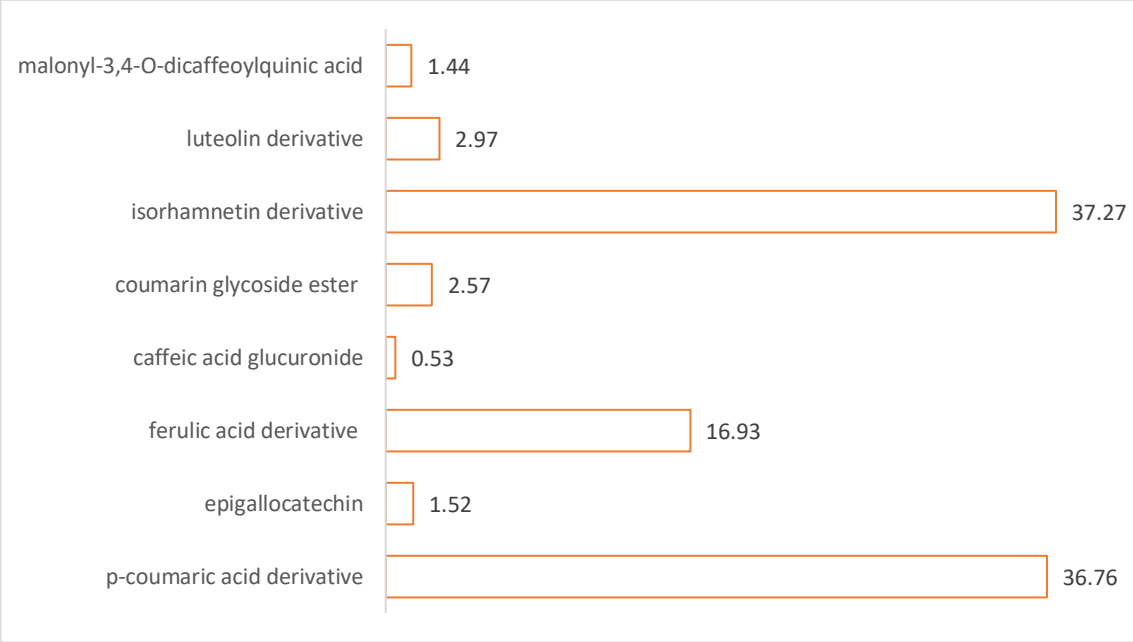

Figure A.7: Relative percentage of the phenolic compounds identified in *Carpobrotus edulis* (Sea fingers)

Appendix 2: Total Phenolic Content by Folin method

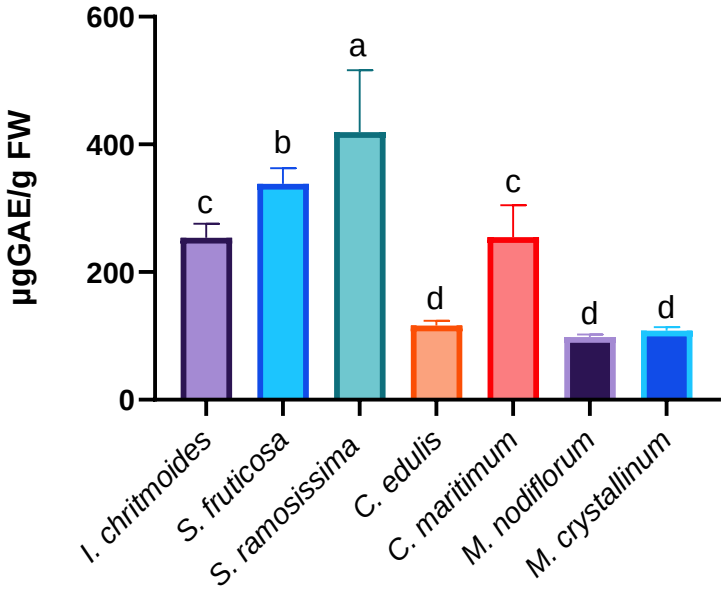

Figure A.8: Total phenolic content of selected halophyte plants by Folin method. The lower-case letters (a to d) denotes significant difference according to Tukey's test ($p < 0.05$). Each column represent average with standard deviation (n=2)

Appendix 3: Chromatogram of digestive fraction

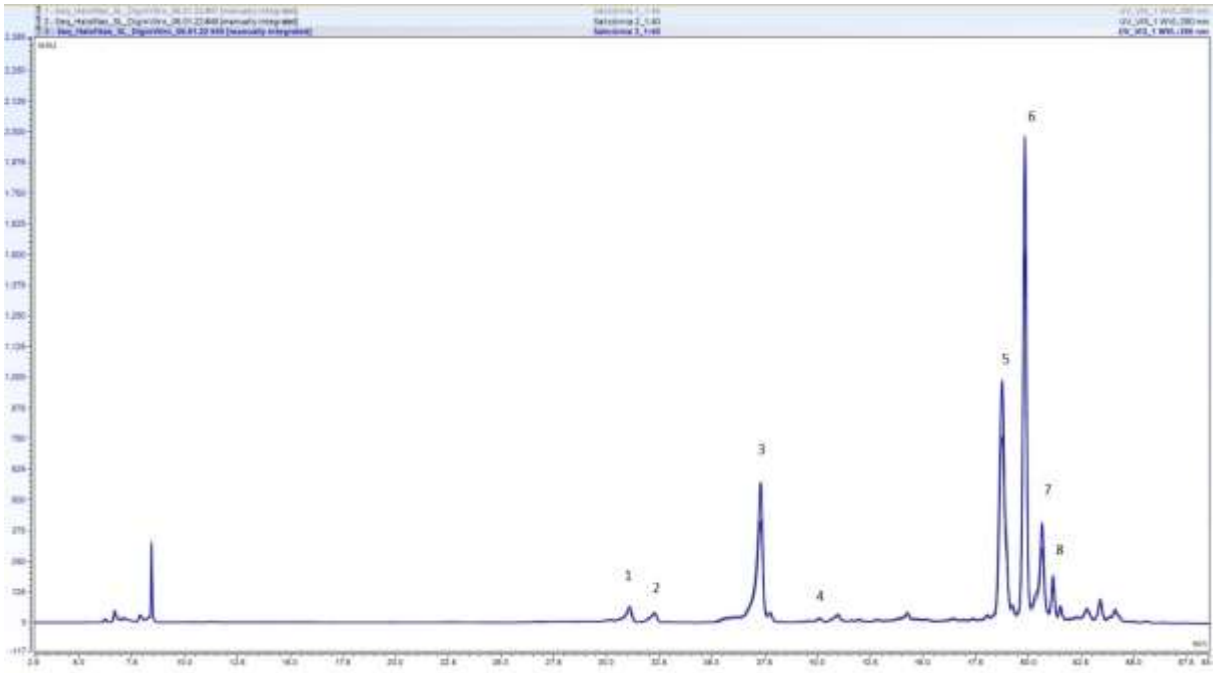

Figure A.9: Cromatrograms of *S. ramosissima* extract; 8 compounds were identified: **1**-neochlorogenic acid; **2**-gallocatechin; **3**-chlorogenic acid; **4**-ferulic acid-glucoside; **5**-3,4-dicaffeoylquinic acid; **6**- 3,5-dicaffeoylquinic acid; **7**- 4,5-dicaffeoylquinic acid and **8**- caffeoyhidrocaffeoylquinic acid at 280 nm

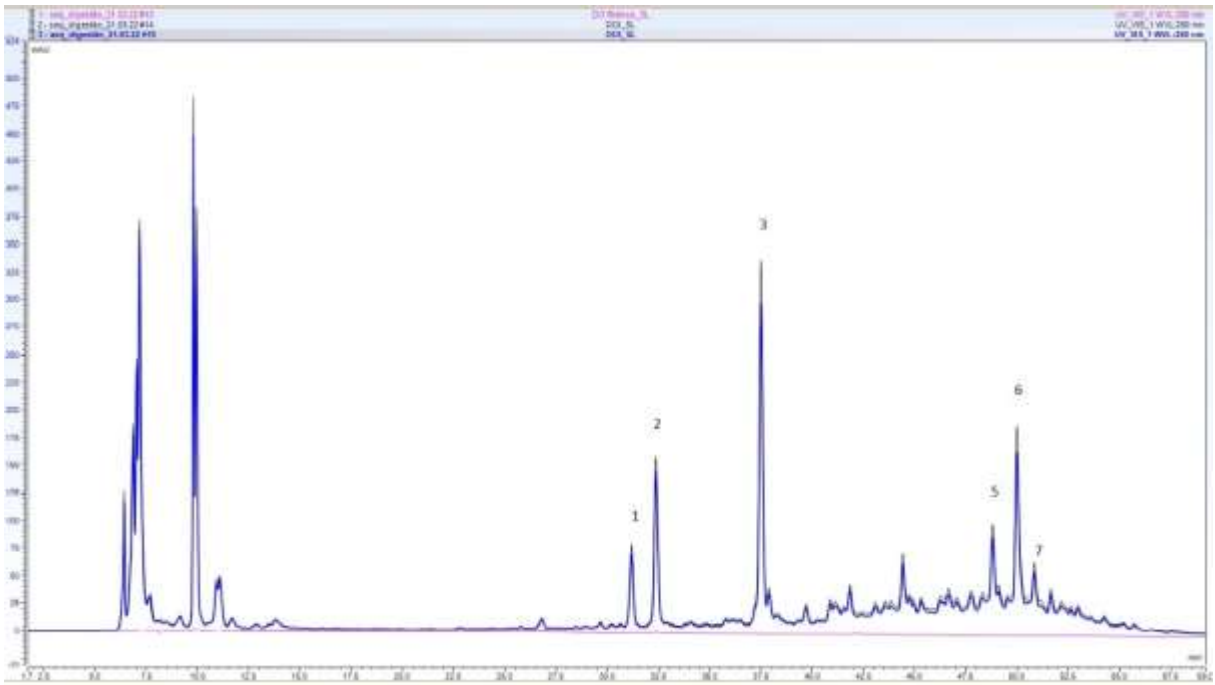

Figure A.10: Cromatrograms of *S. ramosissima* oral *in vitro* digestion phase; 6 compounds were identified: **1**-neochlorogenic acid; **2**-gallocatechin; **3**-chlorogenic acid; **4**-ferulic acid-glucoside; **5**-3.4-dicaffeoylquinic acid; **6**- 3.5-dicaffeoylquinic acid; **7**-4.5-dicaffeoylquinic acid at 280 nm

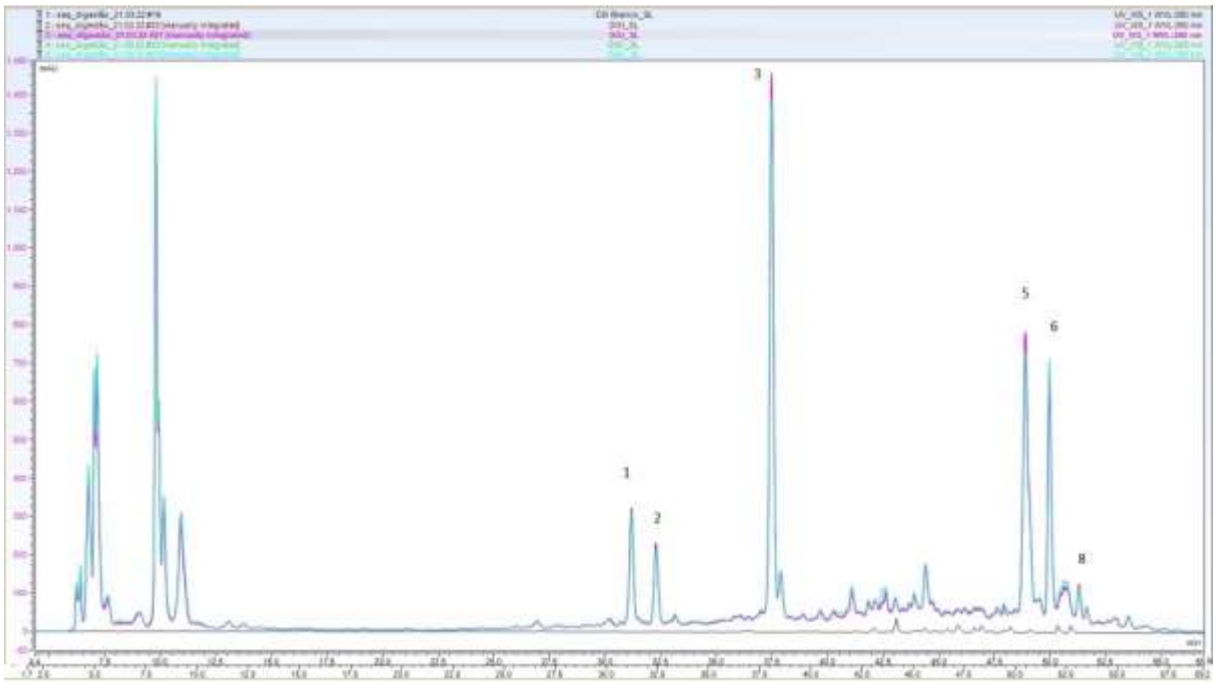

Figure A.11: Cromatograms of *S. ramosissima* gastric *in vitro* digestion phase; 6 compounds were identified: **1**-neochlorogenic acid; **2**-gallocatechin; **3**-chlorogenic acid; **5**-3.4-dicaffeoylquinic acid; **6**- 3.5- dicaffeoylquinic acid; **8**-caffeoylhidrocaffeoyquinic acid at 280 nm

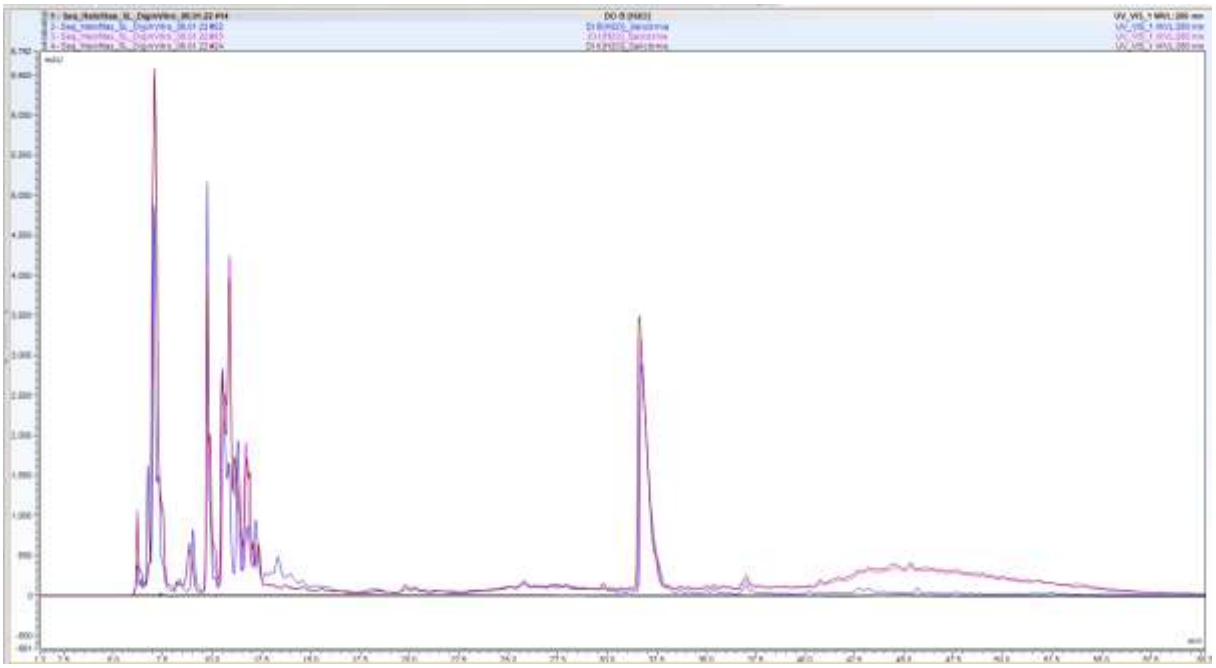

Figure A.12: Cromatograms of *S. ramosissima* intestinal *in vitro* digestion phase; none of the previously identified compounds were identified at this stage at 280 nm

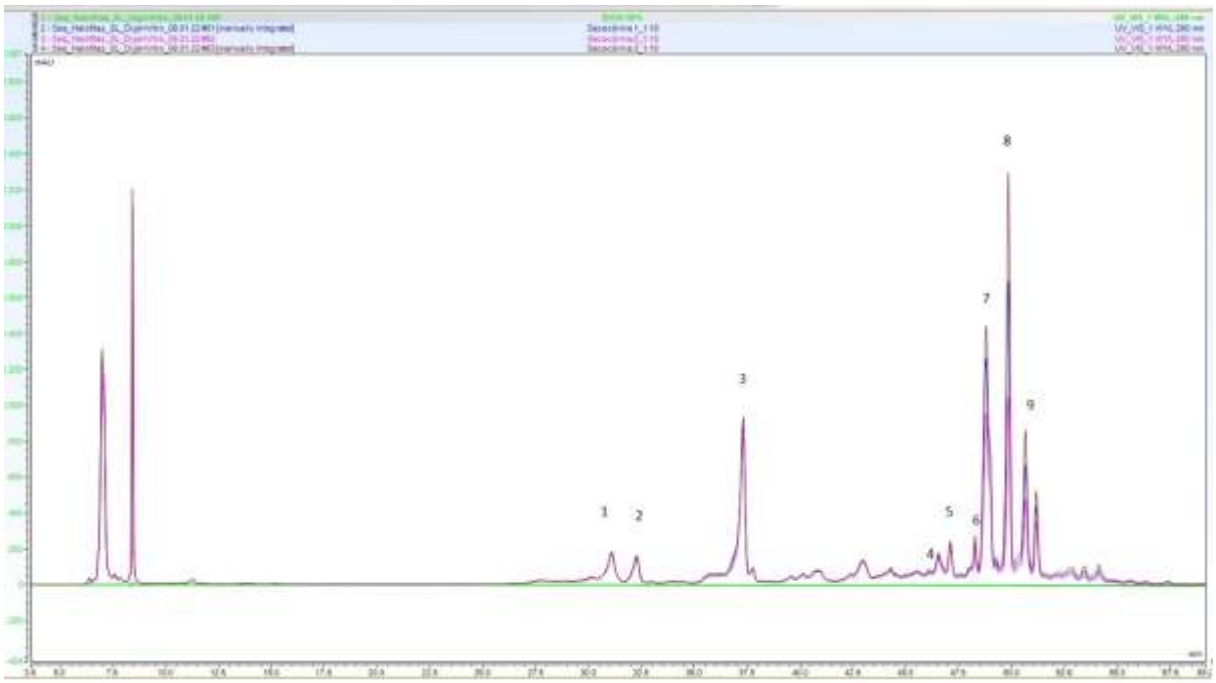

Figure A.13: Cromatrograms of *S.fruticosa* extract; 9 compounds were identified**: 1**-neochlorogenic acid; **2**-gallocate-chin; **3**-chlorogenic acid; **4**-erydictiol-O-hexoside; **5**-rhamnetin hexosyl pentoside ; **6**- Isorhamnetin 3-O-robinoside; **7**-3.4-dicaffeoylquinic acid and **8**- 3.5-dicaffeoylquinic acid and **9**- 4.5-dicaffeoylquinic acid at 280 nm

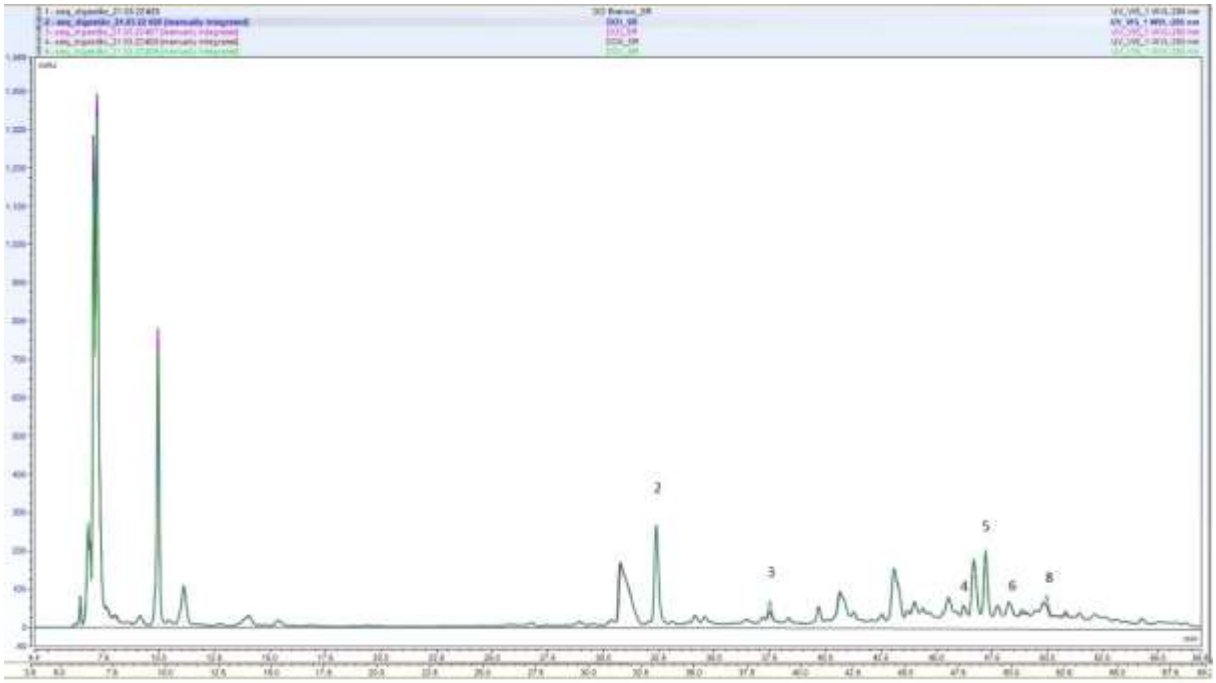

Figure A.14: Cromatograms of *S. fruticosa* oral *in vitro* digestion phase ; 6 compounds were identified: ; **2**-gallocate-chin; **3**-chlorogenic acid; **4**-erydictiol-O-hexoside; **5**-rhamnetin hexosyl pentoside ; **6**- Isorhamnetin 3-O-robinoside; **8**-3,5-dicaffeoylquinic acid ; 4,5-dicaffeoylquinic acid at 280 nm

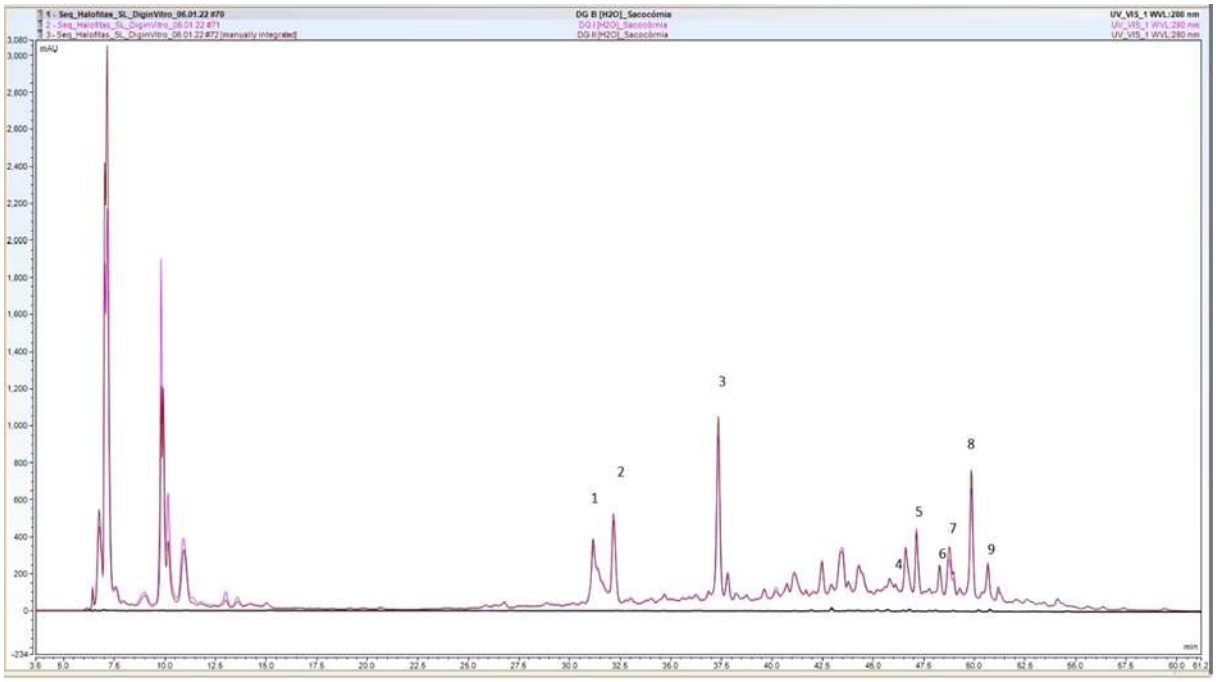

Figure A.15: : Chromatograms of *S. fruticosa* gastric *in vitro* digestion phase 9 compounds were identified: **1**-neochlorogenic acid; **2**-gallocatechin; **3**-chlorogenic acid; **4**-erydictiol-O-hexoside; **5**-rhamnetin hexosyl pentoside ; **6**- Isorhamnetin 3-O-robinoside; **7**-3.4-dicaffeoylquinic acid and **8**- 3.5-dicaffeoylquinic acid and **9**- 4.5-dicaffeoylquinic acid at 280 nm

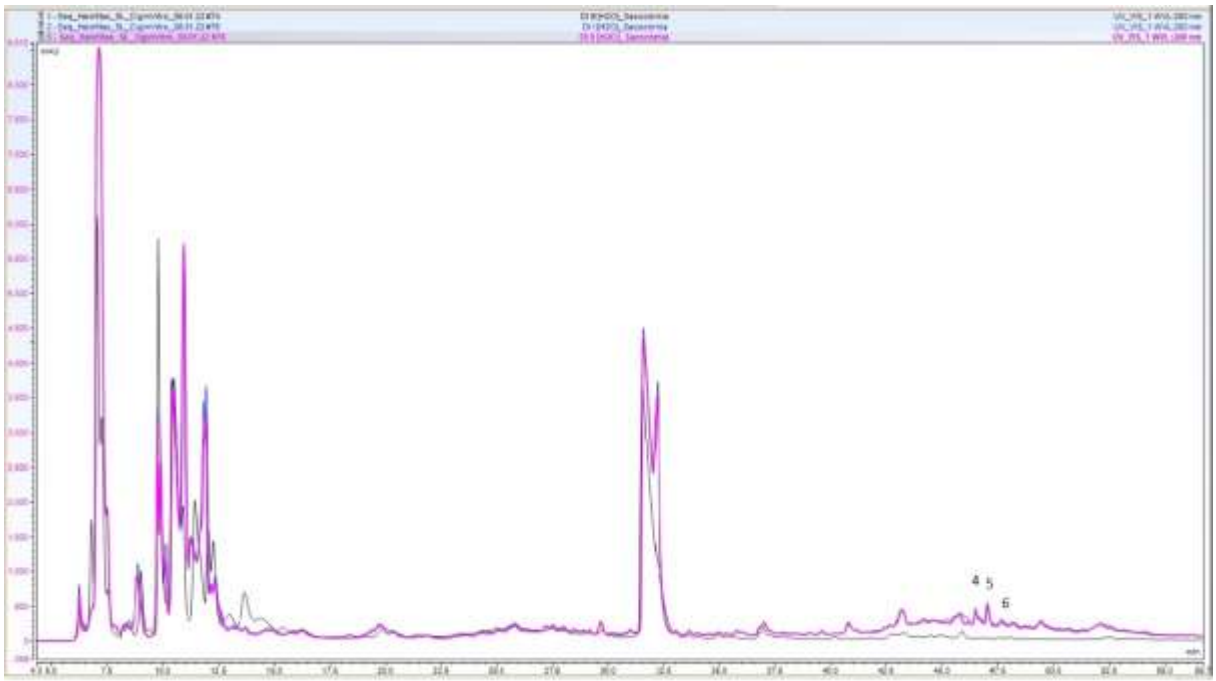

Figure A.16: Chromatograms of *S. fruticosa* intestinal *in vitro* digestion phase; 3 compounds were identified: **4**-erydictiol-O-hexoside; **5**-rhamnetin hexosyl pentoside ; **6**- Isorhamnetin 3-O-robinoside at 280 nm

〈2022〉 João Sousa

IN VITRO BIOACESSIBILITY AND BIOACTIVITY OF PHENOLIC COMPOUNDS FROM HALOPHYTE PLANTS

NOVA SCHOOL OF SCIENCE & TECHNOLOGY