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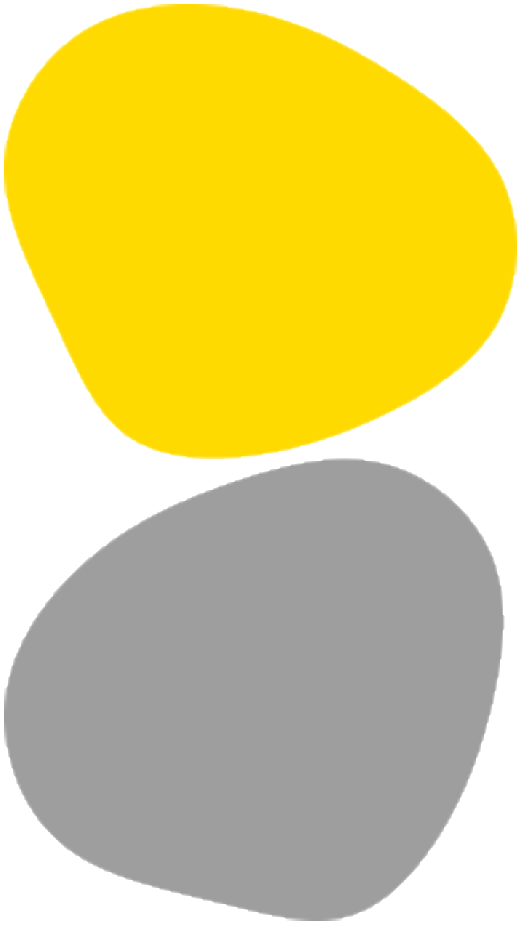
Valorization of the nutraceutical potential of *Salicornia ramosissima* by-product – Impact of gastrointestinal digestion on *in vitro* bioactivity

Inês Maria Manso Santos



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Valorization of nutraceutical potential by *Salicornia ramisissima* by-product – Impact of gastrointestinal digestion on in vitro bioactivity

Autor

Inês Maria Manso Santos

Orientador(es)

Doutora Francisca Rodrigues, PhD, LAQV/REQUINTE – Instituto de Engenharia do Porto

Doutora Diana Pinto, PhD, LAQV/REQUINTE – Instituto de Engenharia do Porto

Doutora Mónica Vieira, PhD, ESS – Escola Superior de Saúde

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Resumo

Este trabalho centra-se na valorização da halófita *Salicornia ramosissima,* já conhecida pelos seus compostos bioativos, através da avaliação do efeito da digestão gastrointestinal no conteúdo fenólico, bioacessibilidade e bioatividade. Com efeito, o principal objetivo deste trabalho foi a avaliação do efeito da digestão gastrointestinal no perfil fitoquímico do subproduto desta halófita, bem como da permeabilidade intestinal dos compostos bioativos. Para isso, foram realizados diversos ensaios para avaliar os teores de fenólicos e flavonoides totais, atividades antioxidante/antiradicalar através dos ensaios FRAP, ABTS e DPPH, capacidade de captação de espécies reativas de oxigénio (ROS), atividade neuroprotetora por inibição da enzima acetilcolinesterase (AChE), viabilidade em linhas celulares intestinais, e efeito dos extratos e das suas frações digeridas na atividade de enzimas antioxidantes e peroxidação lipídica. A permeabilidade intestinal foi avaliada num modelo intestinal de co-cultura composto por células Caco-2 e HT29-MTX por identificação e quantificação dos compostos bioativos que permeam a barreira intestinal através de cromatografia líquida acoplada a espectrometria de massa. Os resultados revelaram a riqueza deste subproduto em compostos fenólicos e flavonoides, comprovando os efeitos da digestão *in vitro* na bioatividade do mesmo. As propriedades antioxidantes e antiradicalares aumentaram durante os diferentes processos da digestão, de modo especial na fase intestinal, com o extrato de subproduto de *S. ramosissima* (SE) a demonstrar maior atividade comparativamente ao subproduto (SP). Adicionalmente, os ensaios enzimáticos – AChE (Acetylcholinesterase), SOD (Superoxide dismutase), CAT (Catalase), GSH-Px (Glutathione peroxidase) e LPO (Lipid peroxidation) – demonstraram benefícios significativos do subproduto de *S. ramosissima* para a saúde humana, evidenciando as suas propriedades neuroprotetora, prevenção da peroxidação lipídica e capacidade de estimular a atividade das diferentes enzimas antioxidantes analisadas. Os ensaios de viabilidade células indicaram que os extratos de SE são seguros para as maiores concentrações estudadas (1000 µg/mL), demonstrando maior viabilidade celular na linha célular HT29-MTX. Os ensaios de permeabilidade intestinal demonstraram que o ácido 4-cafeoilquínico exibiu elevada permeabilidade (34.84%). Este estudo evidencia os potenciais benefícios dos compostos fenólicos de *S. ramosissima* para a saúde humana após o processo digestivo. Os resultados obtidos fornecem uma base sólida para investigações futuras sobre o desenvolvimento de nutracêuticos e alimentos funcionais a partir dos componentes bioativos desta planta. Numa perspetiva futura, estudos subsequentes poderão focar-se na realização de ensaios *in vivo* para confirmar a bioatividade dos compostos digeridos e examinar os seus efeitos na saúde a longo prazo. Além disso, a exploração de outros compostos bioativos presentes em *S. ramosissima* pode ampliar as suas aplicações em contextos terapêuticos.

Palavras-chave: Compostos fenólicos; *Salicornia ramosissima*; atividade antioxidante; digestão gastrointestinal; permeabilidade intestinal; bioacessibilidade; flavonoides; ensaios enzimáticos; espécies reativas de oxigénio.

Abstract

This work focused on the valorization of the halophyte *Salicornia ramosissima*, already known for its bioactive compounds, by evaluating the effect of *in vitro* gastrointestinal digestion on its phenolic content, bioaccessibility, and bioactivity. The main objective of this study was to assess the impact of *in vitro* gastrointestinal digestion on the phytochemical profile of this halophyte by-product, as well as to evaluate their antioxidant and antiradical properties and intestinal permeability of the bioactive compounds. For that, various assays were performed to evaluate the total phenolic and flavonoid contents, antioxidant/antiradical activities by FRAP, ABTS, and DPPH assays, the scavenging capacity of reactive oxygen species (ROS), neuroprotective properties by acetylcholinesterase (AChE) inhibition, intestinal cell viability, and the extracts effect and their digested fractions in the antioxidant enzymes activities and lipid peroxidation. The intestinal permeability was assessed in a Caco-2/HT29-MTX co-culture model by identifying and quantifying the bioactive compounds that permeated the intestinal barrier by liquid chromatography coupled to mass spectrometry. The results revealed the richness of this by-product in phenolic compounds and flavonoids, confirming the effects of the *in vitro* digestion on its bioactivity. The antioxidant and antiradical properties increased throughout the different stages of digestion, particularly after the intestinal phase, with the *S. ramosissima* by-product extract (SE) showing higher activity when compared to the by-product (SP). Additionally, the enzymatic assays – AChE (Acetylcholinesterase), SOD (Superoxide dismutase), CAT (Catalase), GSH-Px (Glutathione peroxidase), and LPO (Lipid peroxidation) demonstrated significant health benefits, highlighting the neuroprotective properties, prevention of lipid peroxidation, and capacity to stimulate the activity of the various antioxidant enzymes studied. Cell viability assays indicated that SE extracts were safe at the highest concentrations studied (1000 μg/mL), demonstrating greater cell viability in the HT29-MTX cell line. The intestinal permeability assays demonstrated that 4-caffeoylquinic acid exhibited the highest permeability (34.84%). This study highlights the potential health benefits of *S. ramosissima* phenolic compounds after the digestion process. The results obtained provide a solid foundation for future research on the development of nutraceuticals and functional foods using the bioactive components of this plant. From a future perspective, subsequent studies will focus on *in vivo* assays to confirm the bioactivity of the digested compounds and investigate their long-term effects on health. Furthermore, exploring other bioactive compounds available in *S. ramosissima* could enhance its applications in therapeutic contexts.

Keywords: Phenolic compounds; *Salicornia ramosissima*; Antioxidant activity; Gastrointestinal digestion; Intestinal permeability; Bioaccessibility; Flavonoids; Enzymatic assays; Reactive oxygen species.

List of publications

- Pinto, D., Santos, I., Teixeira, F., Sut, S., Vieira, M., Salazar, M., Delerue-Matos, C., Dall'Acqua, S., & Rodrigues, F. (2025). *Unraveling the nutraceutical potential of Salicornia ramosissima by-product – Impact of gastrointestinal digestion and intestinal permeability on in vitro bioactivity.* Food Chemistry, 486, 144665. https://doi.org/10.1016/j.foodchem.2025.144665

Índice

Index of Figures

Index of Tables

1. Introdution

1.1. Salicornia ramosissima

Over the years, there has been a constant degradation of agricultural land due to the decrease of fresh water and surface water associated with salinization[1]. Therefore, several studies have been carried out on halophytes due to their higher saline resistance than normal crops[2–4]. Halophytes are plants that can thrive from coastal areas to salt marshes, being subject to multiple environmental stresses, including high levels of salinity, periods of drought, and high exposure to ultraviolet (UV) rays[5]. These plants can survive environmental adversities through adaptive mechanisms that activate the antioxidant enzymes or stimulate the synthesis of secondary metabolites in response to stress[5, 6]. In addition to these characteristics, halophytes can also be used by the pharmaceutical industry in obesity treatments as suppliers of secondary metabolites, such as the flavonoid 3-*O*- β-D-glucopyranoside[7]. *Salicornia* species are widely distributed across temperate and subtropical regions, gaining increasing popularity in the European market in the last years[8]. Particularly, *S. ramosissima* is highly appreciated due to its salty flavor.

Salicornia's name comes from the Latin word "salt" since this plant is one of the most salt-tolerant ones[9]. Within botanical community, *Salicornia* is widely recognized for its appearance, being characterized by the absence of leaves and the presence of succulent-like stalks. Physically, this plant is characterized by several branches that normally reach 35 cm. Dark green is the dominant color, even though it may turn to yellow-green, later pink, or red at the end of its life cycle (Figure 1). The color stems also change according to the saturation of saline stress, turning yellow or red[10].

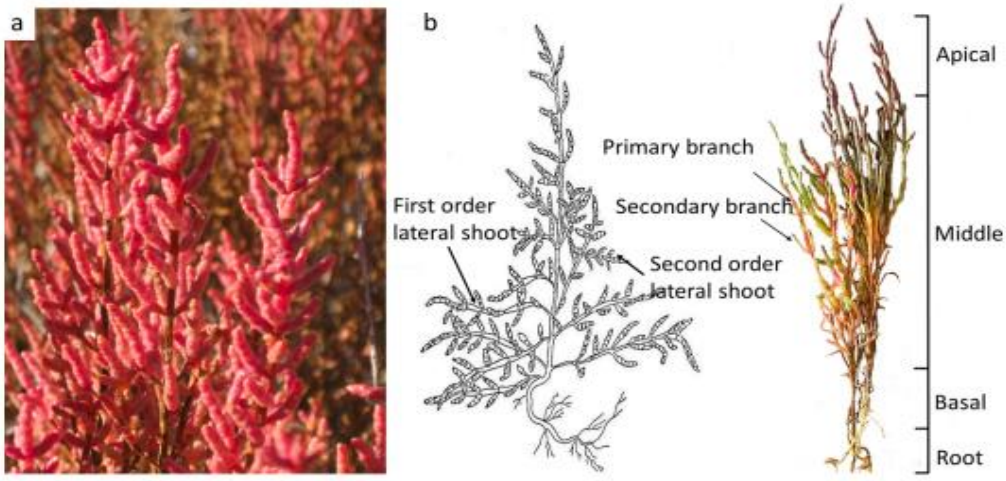

Figure 1 – *Salicornia ramosissima* appearance [11].

The salt stress may lead to the production of reactive oxygen species (ROS) in plants that damage cell membranes and interfere with enzymatic activity[12]. *Salicornia* can resist this stress due to its antioxidant components. Therefore, its phytochemical composition is arousing interest among the scientific community, with studies demonstrating the vast potential of this plant in several areas owing to its antimicrobial, antioxidant, anti-inflammatory, anticancer, antihyperglycemic, antihyperlipidemic, and protective vessel properties[13]. Despite the vast knowledge of plants with high economic potential, there are few studies regarding plants with high tolerance to salinity[13, 14].

In Portugal, *S. ramosissima* is abundantly distributed in salt marshes on the Aveiro estuary and frequently used in local cuisine, particularly as a salt substitute[15]. Once described as the poor man´s asparagus or fishermen´s food [16], this delicacy is fast becoming a trendy garnish in many gourmet restaurants. In Portugal, where *Salicornia* was once considered a weed and a plague, its nutritional properties have recently increased interest in its production and consumption. Moreover, *S. ramosissima* is well-considered by start-up companies, such as Ria Fresh, which was one of the pioneers contributing to its cultivation, production, and incorporation into the health system. They are farmed to the highest quality standards in the Algarve Coast Natural Park and Ria Formosa. This vegetable is planted in December and collected between March and September and its production is done using a flooding system that imitates tides. During the rest of the year, *Salicornia* species became by-product that contain valuable antioxidants, such as polyphenols and natural pigments (namely chlorophylls and carotenoids) which, despite their richness, have yet to be repurposed[17].

1.2. Chemical composition of *S. ramosissima*

1.2.1. Polyphenols

Polyphenols are the largest group of phytochemicals, being widely found in plant-based foods. These bioactive compounds are powerful antioxidants that complement and add functionality to antioxidant enzymes and vitamins, combating the oxidative stress caused by ROS accumulation[18]. Different studies demonstrated that polyphenols subgroups differ in stability, bioavailability, and functions related to human health[19-22]. Sandoval *et al.* [19]described the mechanisms and signaling pathways involved in the metabolic impact of each group of flavonoids on obesity and related disorders, focusing on the liver, white and brown adipose tissue, and central system. The authors concluded that flavonoids are effective in a wide range of obesity-related models, proving that the anti-obesity effects of flavonoids are robust and consistent, with most of the molecular mechanisms underlying these effects being shared by various subclasses of flavonoids[19].

In another study, Márquez Campos *et al.* [20]collected and summarized the available literature on the antidiabetic effects of flavan-3-ol compounds and their microbial metabolites, which are hardly absorbed according to the colonic bacteria activity.

There has been increasing evidence that polyphenols have capacity to perform their actions *in vivo*, although almost studies focused on *in vitro* activity[21, 23].

A diet rich in polyphenols can protect against chronic pathologies, such as cancer, diabetes, and neurodegenerative and cardiovascular diseases, through the modulation of numerous physiological processes, namely cellular redox potential, enzymatic activity, cell proliferation, and their signaling pathways[21, 22]. However, these compounds have low oral bioavailability, due to their biotransformation mediated by enzymes in intestinal and liver cells, as well as by the intestinal microbiota[24]. Despite this, most of the polyphenols demonstrate significant biological effects that have sparked the interest in this low availability/high bioactivity paradox[25].

1.2.2. Polyphenols classification

Currently, more than 8000 phenolic structures are known and around 4000 flavonoids have been identified [26]. Although the chemical definition characterizes polyphenols as compounds that contain phenolic structures, this group of natural molecules is highly diverse and consists of several subgroups. Fruits, vegetables, whole grains, and other types of foods and drinks (such as tea, chocolate, and wine) are rich sources of polyphenols [26]. This vast diversity and distribution of polyphenols has led to the formation of different ways of characterizing these natural compounds. Polyphenols are characterized by their origin, biological function, and chemical structure. In fact, most of the polyphenols present in plants exist in the form of glycosides, with different numbers of sugar units and different positions of acylated sugars in the polyphenol skeleton. Due to all these variables, polyphenols are classified based on the chemical structure of aglycones as shown in Figure 2 [27].

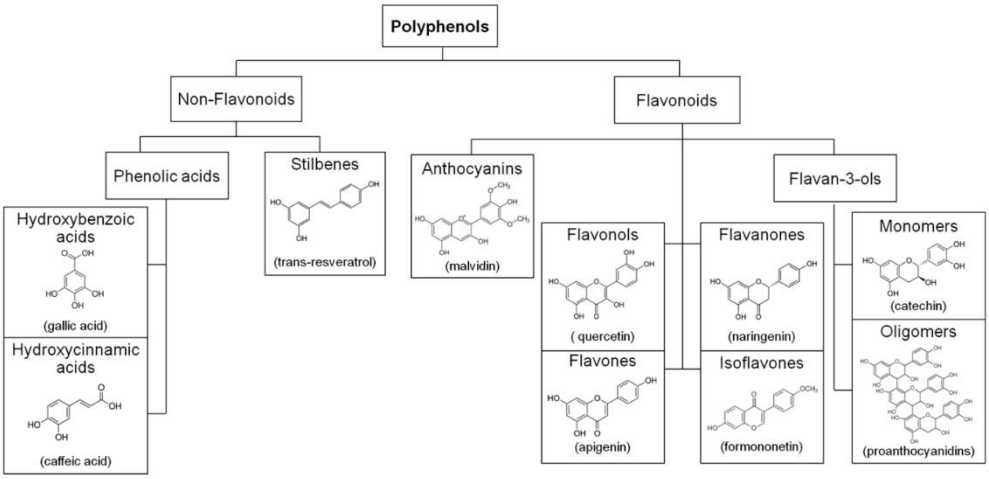

Figure 2 – Polyphenol subgroup hierarchy and structure [28].

1.2.3. Phenolic acids

Phenolic acids are compounds containing in their structure one phenolic ring and at least one carboxylic acid functional group. They can be further classified into two types: benzoic acids and cinnamic acids (Figure 3). Although fruits and vegetables contain many phenolic acids, grains and seeds present them in the conjugated form [29, 30], being only released by hydrolysis mediated via enzymes.

A lot of phenolic acids were discovered in *Salicornia* species [31, 32]. In the first study, *trans*-cinnamic acid was identified in *S. patula* samples from different Spanish geographical regions and harvest seasons, with contents ranging between 20% and 40% of total polyphenols content in interior provinces, with other compounds standing out, namely salicylic acid in the Tinto River coastal saltmarsh in southwestern Iberia and salicylic acid with more than 60% of the content [31]. In comparison, samples from the mainland have no more than 22%. Veratric acid was present in relative concentrations of 40–60% in plant materials from inland provinces, while caffeic acid exhibited the lowest relative concentration (8%) [31]. In another study, the authors identified several other phenolic acids, such as gallic acid (with the highest value of 0.21 mg/g dw for *S. europea*), protocatechuic acid (found in *S. europea*, *S. ramosissima*, and *S. herbacea* with the highest value being 8.4 mg/g dw), chlorogenic acid (detected in all three species with 14.1 mg/g dw being the highest value), and caffeic acid (also found in the three species up to 9.5 mg/g dw) [32]. It is known that these compounds display varied positive biological activities, such as antihypertensive, antimicrobial, reduction of neointimal hyperplasia, antibacterial, amelioration and

prevention of vascular diseases [33–36]. Therefore, the remarkable biological activities of these compounds highlight their potential to promote overall well-being and prevent various health issues.

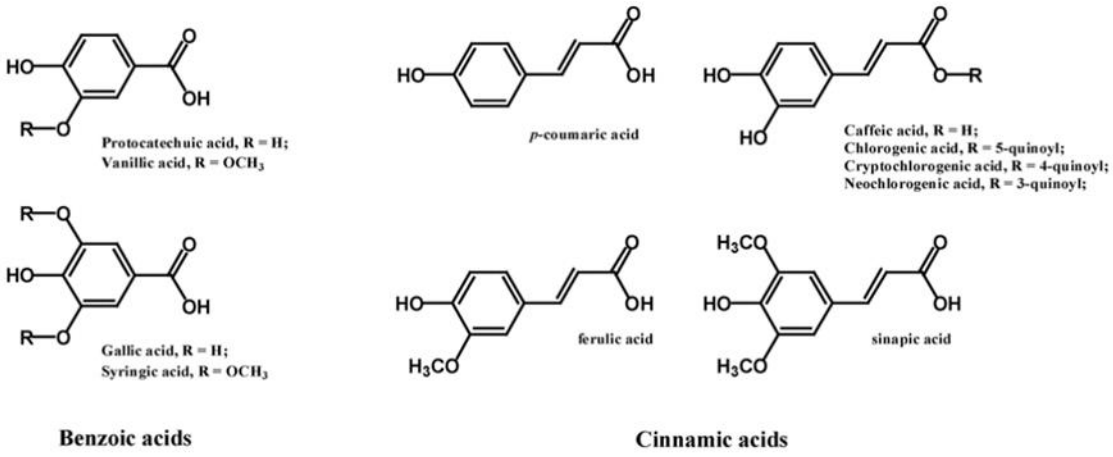

Figure 3 – Phenolic acids subgroups: benzoic acids and cinnamic acids [37].

1.2.4. Flavonoids

Depending on the carbon of the C ring, to which the B ring is attached, as well as the degree of the unsaturation and oxidation of the C ring, flavonoids can be classified into various subgroups, such as chalcones, isoflavones, and neoflavonoids (Figure 4) [36].

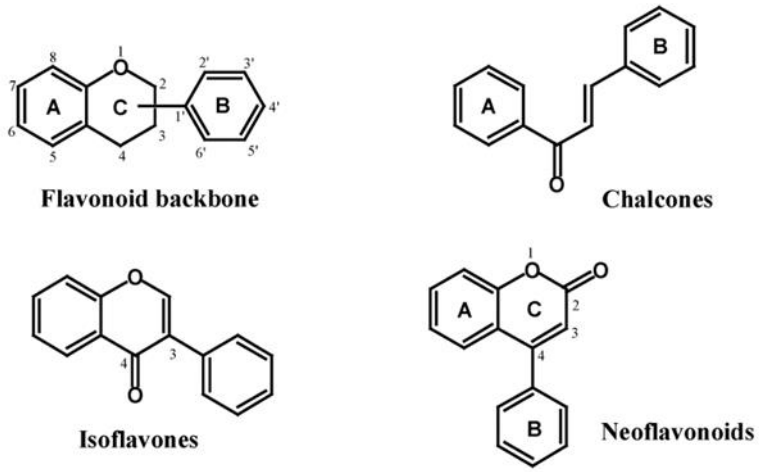

Figure 4 – Flavonoids basic structure and subgroups: isoflavones, chalcones, and neoflavonoids [37].

Isoflavones are flavonoids in which the B ring is bonded to the C ring at position 3. Neoflavanoids are defined as those in which the B ring is joined in position 4, whereas those in which the B ring is linked in position 2 can be further separated into multiple subgroups according to the structural characteristics of the C ring [36]. In plants, these compounds are found mainly as glycosides. The biological activity of these compounds, namely the antioxidant activity, depends on their different structural forms and glycosylation patterns [38]. Rutin hydrate, catechin hydrate, kaempferol, and quercetin were some of the flavonoids identified in some *Salicornia* species [32]. These compounds demonstrate antibacterial and

inhibitory properties of the CYP450 enzyme, as well as capacity to ameliorate and prevent vascular diseases [32, 33, 39].

1.2.5. Polyphenolic amides

Some polyphenols may present functional substituents containing the N group, which is the case of polyphenolic amides. Two groups stand out due to their importance as they are the main components of pepper (capsaicinoids) and oats (avenanthramides) [40]. In addition to the spicy flavor of peppers, capsaicinoids provide strong antioxidant and anti-inflammatory properties, as well as the ability to modulate the cellular oxidative defense system [41]. In the case of avenanthramides, antioxidant activities and inhibitory effects of LDL (low-density lipoprotein) oxidation were demonstrated [40]. Nevertheless, as far as we know, no previous study on *Salicornia* species has identified this polyphenolic class.

1.2.6. Other Polyphenols

In addition to phenolic acids, flavonoids, and phenolic amides, several other polyphenols are constituents of foods considered important for health [37]. Among those already mentioned, resveratrol is present in grapes, red wine, berries, and peanuts [42], while ellagic acid is a dimer of gallic acid and its derivatives found in red fruits (such as strawberries and raspberries) and different nuts' shells [43]. Although ellagic acid and gallic acid are found in their free forms, their glucose esters (a group known as hydrolysable tannins) are also detected in different plants and foods [44].

By high-performance liquid chromatography (HPLC), several chemicals were discovered in *S. ramosissima*, including caffeine, phloretin, and phloridzin [45]. Microwave-assisted extraction (MAE) and conventional maceration (CE), two eco-friendly extraction methods, were used by Silva *et al.* [36] to recover polyphenols from *S. ramosissima* employing water as solvent. Two compounds were found in this *Salicornia* species, namely phloridzin and phloretin [46, 47]. Phloridzin, a phloretin glucoside, is one of the most well-known apple phytochemicals with health-promoting properties, including antidiabetic activity [46], while phloretin is a versatile dihydrochalcone with multiple beneficial effects on human health, such as anticancer, anti-osteoclastogenic, antifungal, and antiviral properties, as well as the ability to increase biological membrane fluidity and drug penetration [47]. While phloretin was exclusively found in the MAE extract, phloridzin was detected in both extracts [36].

Phloridzin was also determined in higher concentrations in a different study conducted by Correia *et al.* [45] employing subcritical water extraction (SWE) at different temperatures. Moreover, caffeine was

more efficiently quantified under moderate temperatures (namely 120 and 140 °C) than at very high ones (180 °C). In addition to activating the central nervous system, caffeine has good effects on the human body since it may help to prevent a variety of chronic disorders with other chemicals [48]. Moreover, phloretin was present in amounts below the method's detection limit [45].

1.3. Biological functions and implications

1.3.1. Antioxidant activity

Polyphenols are secondary metabolites produced by plants to protect themselves from other organisms. Different studies have confirmed the benefits of polyphenols to human health [28, 49, 50]. A high intake of fruits, vegetables, and grains (rich in polyphenols) has been associated with a reduction in the risks of chronic pathologies, including cancer, cardiovascular diseases, chronic inflammation, and degenerative diseases [51]. Recent studies also revealed that many of these diseases are related to oxidative stress and the overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) [52–56].

Polyphenols are powerful antioxidants capable of neutralizing free radicals by donating an electron or a hydrogen atom and, therefore, reduce the rate of oxidation by limiting the production and activation of free radicals. For this reason, polyphenols act as radical scavengers of lipid peroxidation chain reactions (chain breakers) [57]. In addition to scavenging radicals, polyphenols are also known as metal chelators. The chelation of transition metals (such as Fe^{2+}) can directly reduce the Fenton reaction rate, thus avoiding oxidation caused by highly reactive hydroxyl radicals [58]. Polyphenols do not act alone; they can exert antioxidant functions with other compounds, being involved in the regeneration of essential vitamins [59].

Several *in vitro* models have been developed to assess antioxidant activity and, although they are limited in terms of similarity with biological systems, they are often capable of estimating the functionality of polyphenols in these systems and, consequently, their benefits on human health [60]. There are advantages and drawbacks concerning these models. Most of them are simple, quick, and useful procedures to quantify with accuracy the antioxidant potential of the sample under analysis. On the other hand, considering the *in vivo* health relevance, it is imperative to use more than one assay to evaluate the antioxidant activity since the *in vitro* assays can only rank antioxidative activity for their system [60, 61]. From a chemical point of view, polyphenols can potentially induce pro-oxidant activities because, upon receiving an electron or hydrogen atom, they transform into free radicals. Excessive concentration of the substance may pose a potential health risk, although its impact on human health may vary. Therefore, more research in this field is needed to better understand this topic [62].

In addition to the possible antioxidant mode of action, other mechanisms, such as xanthine oxidase inhibition and elevation of endogenous antioxidant levels, are also considered important [63]. Polyphenols can stimulate the activity of antioxidant enzymes, such as glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD), being responsible for the decomposition of hydroperoxides, hydrogen peroxide and superoxide anions, respectively. Polyphenols, such as flavonoids, can be absorbed from the gastrointestinal tract, but due to their rapid metabolism, they have very low plasma concentration levels (less than 1 μmol/L) [62]. This concentration is too low to exhibit significant antioxidant capacity and, therefore, many researchers assume that polyphenols probably do not have any *in vivo* antioxidant activity. Nevertheless, there are only a few studies that evaluated the *in vivo* antioxidant properties of foods and derivatives [64, 65].

S. ramosissima has shown antioxidant properties due to its high polyphenols concentration [17]. By adding halophytes as a new food source for aquafeeds, Marçal *et al.* [17] aimed to boost the productivity and environmental sustainability by incorporating this plant into the diet of a European seabass (*Dicentrarchus labrax*). For two months, the authors fed fish with 2.5, 5, and 10% of this halophyte. The outcomes encouraged the inclusion of the whole-plant biomass up to 10%, replacing wheat meal, showing no impairments in growth performance, improving *D. labrax* oxidative status via regulating GSH-related defense subsystem in a tissue-specific way, and triggering beneficial adaptations of the endogenous antioxidant systems [17]. In another study [14], these antiradical properties are shown by multiple tests, such as ABTS, DPPH, and ROS scavenging assays, probably ascribed to the phytochemicals composition rich in phenolic acids and flavonoids. In addition, the safety of *S. ramosissima* by-product extract was ensured on two dermal cell lines, namely keratinocytes and fibroblasts (HaCaT and HFF-1, respectively). Through this preliminary study, the authors suggested the repurposing of *S. ramosissima* by-product as new potential ingredient for industrial purposes [14].

1.3.2. Beyond the antioxidant activity

Despite the importance of indirect and direct activities related to antioxidant capacity, the functions of these compounds at the cellular level are more complex. There is increasing data supporting that, instead of the relationship they have as electron donors, these compounds exert modulatory actions on cells through protein kinase and lipid signaling pathways [66]. Considering *Salicornia* species, diverse studies attested many biological activities, such as neuroprotective and anti-tumor effects [67, 68]. In a study conducted by Kim *et al.*[69] , *S. europaea* secondary metabolites were isolated and classified. Among them, phytol, a diterpene member of the long-chain unsaturated acyclic alcohols, was identified in this

Salicornia species, exerting many biological functions, such as antimicrobial, cytotoxic, apoptosis- and autophagy-modulating, anticonvulsant, immunomodulatory, antinociceptive, and anti-inflammatory activities [70]. Along with this compound, other interesting phytochemicals were also discovered, namely vanillin, with antidepressant activity [71], and scopoletin, with a variety of pharmacological effects like hepatoprotective capacity [72], PC3 cell proliferation inhibitory effects [73], acetylcholinesterase (AChE) inhibitory capacity [74], as well as hypouricemic [75], antifungal synergistic [76], and antithyroid activities [77].

In another study, Karthivashan *et al.* [78] explored the phytochemical composition and bioactivity of desalted and enzyme-digested *S. europaea* ethanolic extract (SE-EE) using chromatographic techniques and *in vitro* biochemical testing. The authors proved the presence of anti-neuroinflammatory properties and ameliorative effects in microglial cells previously exposed to lipopolysaccharides (LPS) as well as in mice with developed amnesia due to scopolamine content. The results showed that SE-EE has anti-amnesic properties and modulates the behavioral-cum-cognitive impairments in scopolamine-induced AD-like mice (Alzheimer's disease). Furthermore, the anticholinergic, antioxidant, and anti-neuroinflammatory properties of SE-EE contribute to the suppression of complex processes linked to neurodegenerative consequences [78]. Overall, the results pointed out the possibility of developing a dietary supplement using this halophytic plant.

1.4. Nutraceuticals

In 1989, the founding member and chairman of the Foundation for Innovation in Medicine (FIM), Stephen DeFelice, combined the terms "nutrition" and "pharmaceutical" to create the word "nutraceutical" [79]. DeFelice defined nutraceutical as "*a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease*" [80].

The application of nutraceuticals in conventional, non-traditional, fortified, recombinant, phytochemical, herbal, functional foods, dietary supplements, probiotics, and prebiotics has led to different classifications. Depending on their nature, nutraceuticals have a wide range of applications and uses. Since their chemical components and methods of providing health benefits are similar, nutraceutical classification often overlaps. The classifications of nutraceuticals and their health-promotion properties are compiled in Table 1.

Table 1 – Classes of nutraceuticals, examples, active ingredients, and their biological effects.

Class/Type of Nutraceutical	Sources	Active Ingredient	Biological effects	References
		Traditional approaches		
	Tomatoes	Lycopene	Anticancer activity in lungs and prostate, reduce blood pressure	[81]
Functional foods	Salmon	Omega-3	Lower cardiovascular disease and diabetes risk	[82]
	Soy	Saponins	Antioxidant activity, detoxification of enzymes, stimulate immune response, and hormonal metabolism	[83]
	Fermented milk and milk products	*Lactobacillus acidophilus* and *Bifidobacterium spp.*	Prevent gastrointestinal infections and lower cholesterol levels	[84]
	Marine algae	Fucoidans	Antioxidant, anticancer, and anticoagulant activities	[85]
	Broccoli	Sulforaphane and glucosinolates	Decrease risk of several cancers, antioxidant effects	[86, 87]
	Carrots	β -carotene	Reduce cancer risk, improve immune system	[86, 88]
	Aloe	Aloins	Wound healing, antiulcer, anti-inflammatory, immunostimulant, and antimicrobial activities, hematopoietic stimulation	[89, 90]
	Turmeric	Curcumin	Anti-inflammatory and anticarcinogenic effects	[89, 91]
Dietary supplements	Folic acid		Prevent defects in neural tubes, and stimulate red blood cells formation	[89, 92]
	Vitamin A		Antioxidant activity, treat some skin diseases	[93]
	Calcium		Preserve bone, muscles, and teeth nerve health, and prevent osteoporosis	[94, 95]

	Iron		Carry oxygen, and produce energy	[93]
	Vitamin D		Preserve bone, musculoskeletal, and teeth health, and help in calcium absorption	[96]
Probiotics	*Lactobacillus acidophilus*, *Bifidobacterium spp.*, *Streptococcus*, and *Enterococcus*		Improve gut health, replace diarrhea-causing bacteria, anticancer effects	[93, 95, 97]
Prebiotics	Fruto-oligosaccharides		Enhance probiotics growth, and stimulate bifidobacteria proliferation	[98]
	Inulin		Enhance immune system, minerals absorption, and protect bones	[95, 99, 100]
		Non-conventional approaches		
Fortified	Orange juice with calcium	Calcium and ascorbic acid	Glycemic control enhancement, sensitivity to insulin	[101]
	Anthocyanin-fortified bread	Anthocyanin	Reduce digestion rate	[102]
Recombinant	Gold kiwifruit	Ascorbic acid and carotenoids	Strengthening the immune system	[103, 104]

Furthermore, plant-derived nutraceuticals have demonstrated diverse health benefits with few or no side effects, being composed of bioactive components, such as polyphenols, flavonoids, anthocyanins, vitamins, carotenoids, and minerals, with outstanding pro-healthy properties [105].

Gastrointestinal diseases are one of the main causes of mortality and morbidity worldwide, encompassing a recurring concern [106]. These pathologies are directly influenced by diet. In the last years, there has been increasing research into the use of bioactive compounds to resolve or minimize these problems, most of them focusing on the use of natural molecules [51, 81]. Many studies have stated a beneficial relationship between phenolic compounds and health, being crucial to include their intake through the human diet due to their antioxidant, antidiabetic, anti-inflammatory, and even anticancer properties [107].

2. Objectives

The present work focused on the valorization of *S. ramosissima* by-product by exploring the effects of the *in vitro* gastrointestinal digestion and intestinal permeability on the bioaccessibility and bioactivity of the phenolic compounds extracted. The specific objectives undertaken in the present thesis were:

- To extract bioactive compounds from *S. ramosissima* by-product by a green conventional technique;
- To evaluate the *in vitro* antioxidant/antiradical properties (by ABTS, DPPH, and FRAP assays) of the undigested extract and by-product and respective digested fractions;
- To assess the scavenging capacity of the undigested extract and by-product and respective digested fractions against ROS;
- To identify and quantify the phenolic compounds in the undigested extract and by-product upon *in vitro* digestion by HPLC;
- To evaluate the cell viability of *S. ramosissima* by-product extract in two intestinal cell lines, namely Caco-2 and HT29-MTX;
- To identify and quantify the bioactive compounds that permeate the intestinal co-culture model composed by Caco-2/ HT29-MTX;
- To evaluate the neuroprotective properties of the undigested extract and by-product and the respective digests by AChE inhibition;
- To assess the antioxidant enzymes activities and protective effects against lipid peroxidation in the presence of the undigested extract and the by-product and respective digests.

3. Materials and Methods

3.1. Chemicals and reagents

All chemicals, reagents, solvents, and standards are of analytical reagent grade, used as received or dried by standard procedures, and acquired from commercial sources. 123-Dihydrorhodamine (DHR), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), gallic acid, sodium acetate, sodium carbonate (Na_2CO_3), catechin, aluminum chloride ($AlCl_3$), nitrotetrazolium blue chloride (NBT), fluorescein, sodium hydroxide (NaOH), phenazine methosulfate (PMS), sodium nitrite ($NaNO_2$), potassium persulfate, Folin-Ciocalteu's reagent, and Trolox were supplied by Sigma-Aldrich (Steinheim, Germany). 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), β-nicotinamide adenine dinucleotide (NADH), and ferrous sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, ferric chloride, anhydrous absolute ethanol, potassium phosphate monobasic (KH_2PO_4), and hydrogen peroxide were acquired from Merck (Darmstadt, Germany).

The commercial kits used to evaluate the antioxidant enzymes activities, namely GSH-Px and SOD, and lipid peroxidation (LPO) were supplied by Sigma-Aldrich (Steinheim, Germany). The enzymatic kits for CAT and AChE were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), non-essential amino acids, penicillin, streptomycin, and trypsin-EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Dimethylsulfoxide (DMSO) was acquired from AppliChem (Darmstadt, Germany).

3.2. Samples

S. ramosissima by-product (SP) was kindly provided by RiaFresh (Faro, Portugal) in March 2021. After being dehydrated for 24 h at 41 °C (Excalibur Food Dehydrator, USA), samples were ground to 1 mm particle size in a grinder (Moulinex A320, France) and stored at room temperature (± 20 °C) in the dark until further extraction as seen in Figure 5.

Figure 5 – Dehydrated *Salicornia ramosissima* by-product (left) and dried powdered by-product (right).

3.3. Preparation of *S. ramosissima* by-product extract (SE)

S. ramosissima by-product extract (SE) was prepared by conventional extraction at 80 °C for 10 min under constant agitation (200 RPM) using water as solvent and following the optimizing study performed by our research team [14]. The extracts obtained were filtered through Whatman nº 1 paper, frozen at -80 °C, lyophilized (Telstar, model Cryodos -80, Spain), and kept at 4 °C until further experiments.

3.4. *In vitro* simulated gastrointestinal digestion

The *in vitro* simulated digestion was performed in subsequential phases and carried out using the methodology described by Minekus *et al.* [108], with minor modifications [109]. The SE was previously dissolved in distilled water at a concentration of 50 mg/mL. For the oral digestion, powdered SP (1 g) and SE solution (50 mg/mL) were mixed with simulated salivary fluid at 1:1 (*w/v*) ratio containing 75 U/mL salivary α-amylase at pH 7. The mixture was then incubated at 37 °C for 2 min in a water bath (Ovan, BSC127-E model, Barcelona, Spain) with stirring. Aliquots of the oral digest were taken after 2 min.

The residual oral digest was combined with simulated gastric fluid in a 1:1 (*v/v*) ratio, containing 2000 U/mL of pepsin at pH 3, and the mixture was stirred for 2 h at 37 °C in a water bath. Aliquots of the gastric digest were taken after 2 h.

For the intestinal digestion, the remaining gastric digest was merged with simulated intestinal fluid at a 1:1 (*v*/*v*) ratio containing 100 U/mL pancreatin and 10 mM bile at pH 7. After that, the mixture was stirred while being incubated for 2 h at 37 °C in a water bath. Aliquots of the intestinal digest were taken after 2 h. The aliquots collected at the end of each digestion phase were then centrifuged at 10,000× *g* for 10 min and stored at -80 °C.

KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, and $(NH_4)_2CO_3$ were used to prepare the simulated fluids. Two independent experiments were performed for each phase. The phenolic recovery rates were determined using the following Equation (1):

$$\text{Recovery (\%)} = (PC_{DS}/PC_{FC}) \times 100 \quad (1)$$

Where PC_{DS} refers to the phenolic content in the digested fractions and PC_{FC} refers to the phenolic content in the undigested extract. The bioaccessibility (%) corresponds to the recovery rate after all digestion phases. The digestion procedure is summarized in Figure 6.

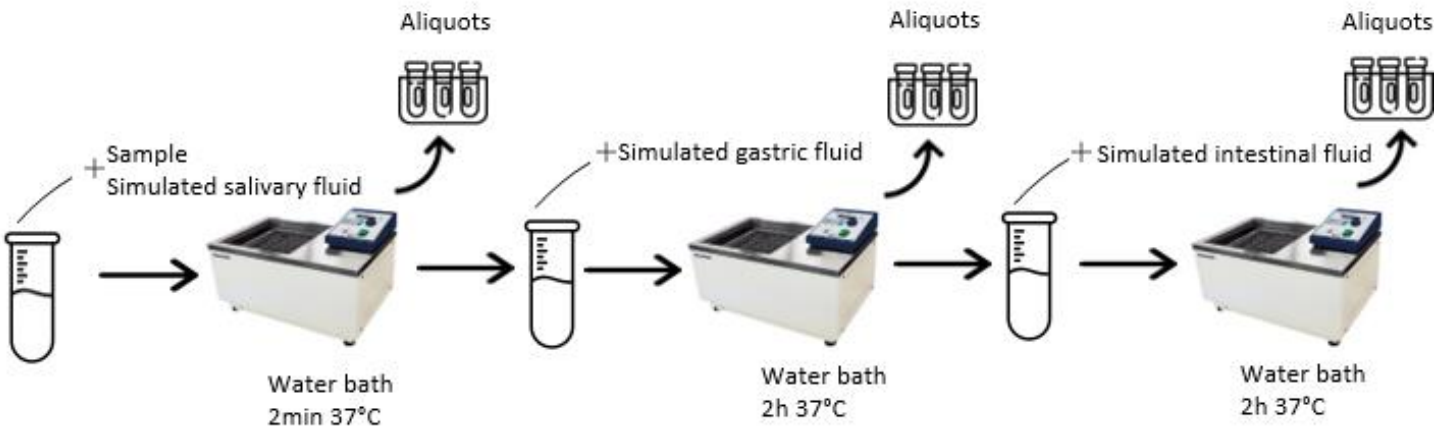

Figure 6 – Scheme of the in vitro simulated gastrointestinal digestion procedure applied in this study.

3.5. Preparation of bioactive compounds from *S. ramosissima* by-product (SP)

Powdered dried SP (1 g) was extracted with distilled water (50 mL) into a test tube and then placed in a sonicator (Sonorex Super, Bandelin, Germany) for 10 min and centrifuged at 10,000× *g* for 10 min. The extract was filtered and stored at 4 °C until further analysis.

3.6. Quantification of the biactive compounds

3.6.1. Determination of Total Phenolic Content

The Total Phenolic Content (TPC) was determined according to the methodology described by Costa *et al.* [110], with minor modifications [109]. The original digested samples were all diluted at 1:4 (*v/v*), except the oral digest from SP (SPO) which was diluted at 1:8 (*v/v*). The undigested samples were diluted at 1:8 (*v/v*) and 1:4 (*v/v*), respectively, for SP and SE. Briefly, an aliquot of 30 µL of each sample was added to 150 µL of Folin-Ciocalteu's reagent (1:10, *v/v*) and 120 µL of 7.5% (*w/v*) Na_2CO_3 solution in a 96-well microplate. Thereafter, the microplate was incubated in a microplate reader (BioTek Instruments, Synergy HT, VT, USA) for 15 min at 45 ºC and cooled for 30 min at room temperature in the dark. The absorbance was measured at 765 nm (Figure 7). The calibration curve was prepared with gallic acid, working as standard (concentrations = 5-100 µg/mL; $R^2 \geq 0.9987$). The assays were made in triplicate. The results were expressed as micrograms of gallic acid equivalents (GAE) per 100 milligrams of extract on dry weight (dw) (µg GAE/100 mg dw).

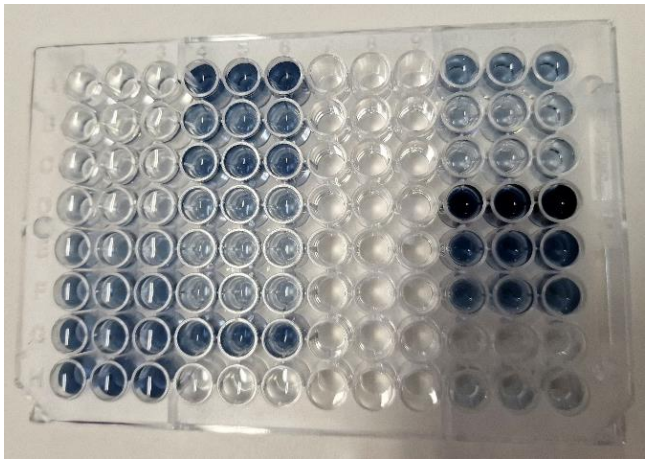

Figure 7 – Total Phenolic Content microplate

3.6.2. Determination of Total Flavonoid Content

The Total Flavonoid Content (TFC) was determined according to the methodology described by Costa *et al.* [110], with minor modifications [109]. The original digests SP and SE were all diluted at 1:2 (*v/v*). SE was previously diluted at 1 mg/mL using distilled water. Briefly, an aliquot of 30 µL of each sample was added to 75 µL of distilled water and 45 µL of 1% (*w/v*) $NaNO_2$ solution. After 5 min, 45 µL of 5% (*w/v*) $AlCl_3$ was added and, after 1 min, 60 µL of 1 M NaOH and 45 µL of distilled water were also added. Thereafter, the absorbance was measured at 510 nm using the microplate reader described above (Figure 8). The calibration curve was prepared with catechin, working as standard (concentrations =

5-300 μg/mL; R^2 ≥ 0.9984). The assays were made in triplicate. The results were expressed as micrograms of catechin equivalents (CE) per 100 milligrams of extract on dw (μg CE/100 mg dw).

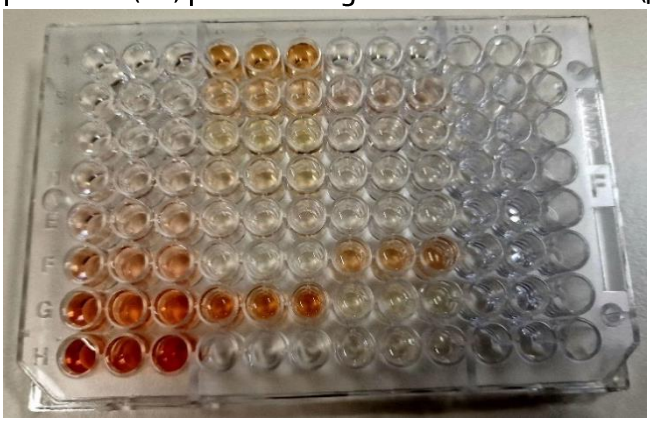

Figure 8 – Total Flavonoids Content microplate.

3.7. *In vitro* antioxidant/antiradical activities

3.7.1. Ferric Reducing Antioxidant Power Assay

The Ferric Reducing Antioxidant Power Assay (FRAP) was evaluated according to Benzie and Strain [111], with minor modifications [109]. This method is conducted in an acidic medium, being based on the reduction of ferric 2,4,6-tripyridyl-*s*-triazine complex (Fe (III)-TPTZ) to the ferrous colored form (Fe (II)-TPTZ) in the presence of antioxidants. The original digests were all diluted at 1:4 (*v*/*v*), except the SPO which was diluted at 1:20 (*v*/*v*). The undigested samples were diluted at 1:4 (*v*/*v*) and 1:20 (*v*/*v*), respectively, for SE and SP. The reaction was carried out in a 96-well microplate. Each well contained 35 μL of sample and 265 μL of FRAP reagent, which was previously prepared with 75 mM sodium acetate buffer, 7.5 mM TPTZ solution, and 7.5 mM $FeCl_3.6H_2O$ solution at 10:1:1 (*v*/*v*/*v*) ratio. The reaction mixture was measured spectrophotometrically at 595 nm using the microplate reader described above, after incubation at 37 ºC for 30 min (Figure 9). The assays were made in triplicate. The calibration curve was prepared with ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), corresponding to the standard solution (concentrations = 25-500 μmol/L; R^2 ≥ 0.9997). The results were expressed as micrograms of ferrous sulfate equivalents (FSE) per 100 milligrams of extract on dw (μg FSE/100 mg dw).

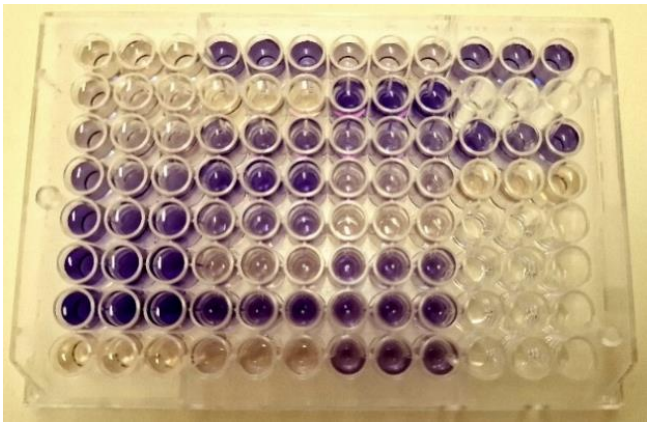

Figure 9 – Microplate of FRAP assay.

3.7.2. $ABTS^{\bullet+}$ scavenging activity assay

The ABTS radical-scavenging activity of samples was determined using the method of Re *et al.*[112]. The original digests were diluted at 1:4 (*v*/*v*), except SPO and the gastric digest from SP (SPG), which were tested at dilutions 1:20 (*v*/*v*) and 1:8 (*v*/*v*), respectively. The undigested samples were diluted at 1:4 (*v*/*v*) and 1:20 (*v*/*v*), respectively, for SE and SP. Firstly, 7 mM ABTS and 2.45 mM potassium persulfate stock solutions were prepared. Subsequently, equal volumes of the two stock solutions were added, resulting in the ABTS cationic stock solution, which was left for 16 h in the dark, at room temperature. The ABTS cationic stock solution was filtered and diluted in ultrapure water until obtaining an absorbance of 0.700 ± 0.02 at 734 nm. Afterwards, in a 96-well microplate, 20 µL of samples were added to 180 µL of the ABTS radical working solution, followed by a posterior incubation period of 6 min in the dark, at room temperature. Finally, the absorbance was measured at 734 nm in the microplate reader described above (Figure 10). The assays were made in triplicate. An ascorbic acid calibration curve was prepared (concentrations = 5-100 µg/mL; $R^2 \geq 0.9986$), and the results were expressed in micrograms of ascorbic acid equivalents (AAE) per 100 milligrams of extract on dw (µg AAE/100 mg dw).

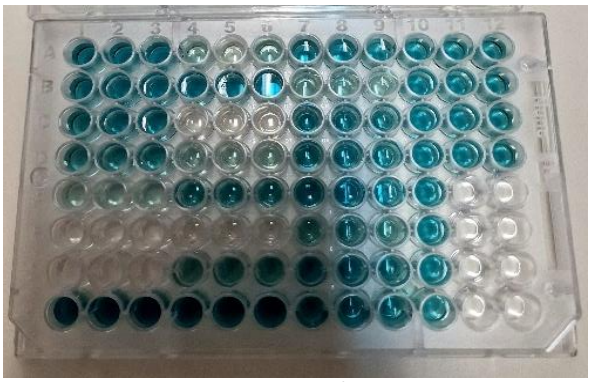

Figure 10 – Microplate of ABTS assay.

3.7.3. DPPH· scavenging activity assay

The DPPH radical scavenging ability was assessed according to the procedure described by Barros *et al.*[113]. The original digests were all diluted at 1:4 (*v*/*v*), except SPO and SPG which were diluted at 1:20 (*v*/*v*) and 1:8 (*v*/*v*), respectively. The undigested samples were diluted in concentrations of 1:4 (*v*/*v*) and 1:20 (*v*/*v*), respectively, for SE and SP. In a 96-well microplate, 30 µL of samples were added to 270 µL of DPPH solution (6×10^{-5} M), which contains DPPH radicals dissolved in absolute ethanol. Then, the absorbance was measured at 525 nm in the microplate reader described above for 40 min (Figure 11). The assays were made in triplicate. Trolox was used as standard to elaborate the calibration curve (concentrations = 5–175 µg/mL; $R^2 \geq 0.9960$). The results were expressed as micrograms of Trolox equivalents (TE) per 100 milligrams of extract on dw (µg TE/100 mg dw).

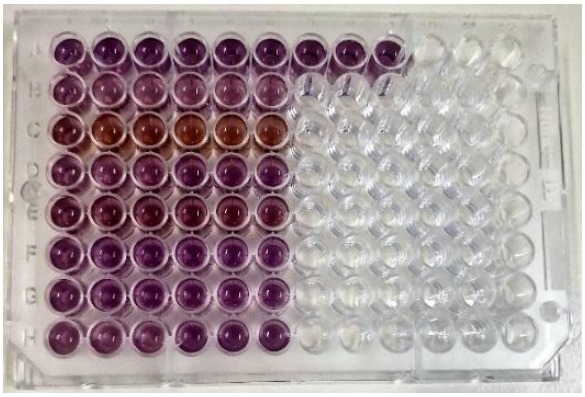

Figure 11 – Microplate of DPPH assay.

3.8. Reactive oxygen species scavenging capacity assays

3.8.1. Superoxide Radical Scavenging Assay

The superoxide anion radical ($O_2^{\cdot-}$) scavenging activity was evaluated based on the reduction of NBT into purple-colored diformazan mediated by $O_2^{\cdot-}$ generated through the non-enzymatic NADH/PMS/O_2 system, according to Gomes *et al.*[114]. The ability to capture the $O_2^{\cdot-}$ was spectrophotometrically determined in 96-well microplates. Six different concentrations were tested for the samples, corresponding to concentration ranges of 31.25–1000 µg/mL and 3.13–100 µg/mL, respectively, for SE and SP prepared with 19 mM phosphate buffer at pH 7.4. The original digests were tested directly without dilution. Briefly, in each well, 50 µL of samples, 50 µL of NADH (166 µM), 150 µL of NBT (43 µM), and 50 µL of PMS (2.7 µM) were added. Gallic acid was used as positive control (concentrations range = 3.91–125 µg/mL). The absorbance was measured in the microplate reader described above at 560 nm for 6

min (Figure 12), and the results were expressed as the inhibition, in IC_{50} (μg/mL) or percentage, of the NBT reduction to diformazan.

Figure 12 – Microplate of the superoxide anion radical scavenging assay

3.8.2. Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide (H_2O_2) scavenging capacity was evaluated by the methodology validated by Müller [115], with minor alterations, in a medium containing ABTS and peroxidase. The 1 U/mL peroxidase solution was prepared with 0.1 M phosphate buffered saline (PBS) solution at pH 5. Six different concentrations of SE (31.25–1000 μg/mL) and SP (3.13–100 μg/mL) were tested after dissolved with the respective PBS solution, while the original digests were tested directly. Briefly, in a 96-well microplate, 20 μL of samples or positive control, 100 μL of 0.1 M PBS solution, and 20 μL of 10 mM H_2O_2 were added to each well. The microplate was incubated at 37 °C for 5 min, and then 30 μL of 1.25 mM ABTS and 30 μL of 1 U/mL peroxidase were added. The microplate was again incubated at 37 °C for 10 min and the absorbance was read at 405 nm. The assays were performed at least in triplicate. Gallic acid was used as positive control (concentrations range = 31.25–1000 μg/mL). The results were exhibited as the inhibition, in IC_{50} (μg/mL) or percentage, of the ABTS oxidation mediated by H_2O_2 and catalyzed by horseradish peroxidase.

3.8.3. Peroxyl Radical Scavenging Assay

The oxygen radical absorbance capacity (ORAC) assay measures the fluorescence signal resulting from the oxidation of fluorescein mediated by peroxyl radical ($ROO^{\cdot}$). The presence of antioxidants inhibits or scavenges the $ROO^{\cdot}$ produced in the medium, hindering the fluorescence decay. This capacity was measured according to Gomes *et al.* [114]. Six different concentrations of SE (15.63–500 μg/mL) and SP (0.78–25 μg/mL) were tested after being dissolved with the respective PBS solution. The SE and SP digestion samples were tested at the dilutions 1:100 (*v/v*) and 1:50 (*v/v*) for the oral, gastric, and intestinal digests. In a 96-well microplate, 150 μL of 61.2 nM fluorescein, 25 μL of sample or positive controls, and

25 µL of 19.1 mM AAPH were added to each well. After that, the microplate was incubated for 2 h in the microplate reader described above and the fluorescence intensity was measured each minute, at the excitation/emission wavelengths of 485±20/528±20 nm. Gallic acid was used as positive control and Trolox was used as standard (both tested in a concentrations range between 0.05 and 1.50 µg/mL). The results were expressed as micrograms of Trolox equivalents (TE) per milligram of extract on dw (µg TE/mg dw).

3.8.4. Hypochlorous Acid Scavenging Assay

The hypochlorous acid (HOCl) scavenging assay was performed according to Gomes *et al.*[114]. The methodology was based on HOCl-mediated oxidation of DHR to rhodamine. The HOCl solution was prepared using a 5 µM NaOCl solution and adjusting the pH value to 6.2 by adding H_2SO_4 solution. The HOCl concentration was calculated according to the mean of spectrophotometric readings at 235 nm with the molar absorption coefficient of 100 $M^{-1}cm^{-1}$. The HOCl working solution was prepared by dilution in 100 mM phosphate buffer at pH 7.4. Six different concentrations of SE (1.95–62.50 µg/mL) and SP (31.25–1000 µg/mL) were prepared using the respective phosphate buffer solution and tested. The assays were performed at least in triplicate. Briefly, in a 96-well microplate, 150 µL of 100 mM phosphate buffer, 50 µL of sample or positive controls, 50 µL of 5 µM DHR, and 50 µL of 5 µM HOCl were added to each well. Gallic acid was used as positive control in a concentrations range between 0.24 and 7.81 µg/mL. The fluorescence was read in the microplate reader described above at the excitation/emission wavelengths of 485±20/528±20 nm at 37 °C for 5 min. The results were exhibited as the inhibition, in IC_{50} (µg/mL) or percentage, of the DHR oxidation mediated by HOCl.

3.9. Enzymatic Assays

3.9.1. Acetylcholinesterase Activity Assay

The AChE activity assay was performed using a commercial kit (MAK119, Sigma-Aldrich, St. Louis, MO, USA). This kit allows us to measure the capacity of our samples and digests to inhibit AChE activity. The samples were diluted at 1:50 (*v*/*v*) and 1:100 (*v*/*v*) in the respective assay buffer. In a 96-well microplate, 10 µL of sample and 190 µL of working reagent were added to each well. Distilled water was used for the blank and the assay calibrator was used for the positive control. The microplate was then incubated for 2 min at room temperature and then the absorbance was read in the microplate reader described above at 412 nm. The absorbance was also read after 10 min at the same wavelength. The results were exhibited as percentage (%) of AChE inhibition.

3.9.2. Superoxide Dismutase Activity Assay

The SOD activity assay was performed using a commercial kit (Sigma-Aldrich, Steinheim, Germany). This kit allows us to measure the SOD activity. In a 96-well microplate, 20 µL of sample, 200 µL of WST working solution, and 20 µL of enzyme working solution were added to each well. For the blank, ultrapure water was used. SOD solution was used for the preparation of the calibration curve. The microplate was incubated for 20 min at 37 °C. The absorbance was read in a microplate reader at 450 nm. The results were exhibited as percentage (%) of SOD inhibition.

3.9.3. Catalase Activity Assay

The CAT activity assay was performed using a commercial kit (Merck, Darmstadt, Germany). This kit allows us to estimate the CAT activity by spectrophotometric measurement of formaldehyde produced in the reaction with methanol. In a 96-well microplate, 20 µL of sample, 100 µL of assay buffer, and 30 µL of methanol were added to each well. Then, 20 µL of diluted H_2O_2 was added to all the wells containing samples, standards, and controls to initiate the reaction. The microplate was incubated on a shaker for 20 min at room temperature. After that, 30 µL of potassium hydroxide was added to terminate the reaction, followed by 30 µL of Purpald® (as chromogen). The microplate was again incubated for 10 min at room temperature on a shaker. Finally, 10 µL of potassium periodate was added to each well of the microplate and incubated for 5 min on a shaker before its reading at 540 nm in a microplate reader. Formaldehyde was used for the preparation of the standard curve, and CAT was used as positive control. The results were exhibited as CAT activity (nmol/min/g dw).

3.9.4. Glutathione Peroxidase Activity Assay

The GSH-Px activity assay was performed using a commercial kit (Sigma-Aldrich, Steinheim, Germany). This kit allows us to indirectly measure the GSH-Px activity through the decrease of NADPH absorbance. In a 1 mL quartz cuvette, 680 µL of assay buffer, 37.5 µL of NADPH reagent, 25 µL of sample, and 7.5 µL of *tert*-butyl hydroperoxide solution were added. The absorbance was read at 340 nm in a double beam spectrophotometer after 15 seconds and then every 10 seconds with a total number of six readings. For the blank, the sample volume was replaced by assay buffer. The results were exhibited as GSH-Px activity (U/g *dw*).

3.9.5. Lipid Peroxidation Activity Assay

The LPO activity assay was performed using a commercial kit (Sigma-Aldrich, Steinheim, Germany). This kit allows us to measure the formation of malondialdehyde (MDA) as product of LPO which is proportional to the fluorescence emitted. In glass test tubes, 125 µL of sample was added to 375 µL of thiobarbituric acid (TBA) solution and the mixture was incubated for 60 min at 95 °C. After that, 200 µL of the solution obtained was transferred to each well of a 96-well microplate. The absorbance was read at 532 nm. The results were exhibited as MDA concentration (nmol MDA/g dw).

3.10. Cell Viability Assays

3.10.1. Cell lines, primary isolation, and culture conditions

Caco-2 (ATCC Number: HTB-37; Caucasian; 72 years; male; colon) and HT29-MTX (ATCC Number: HTB-38; Caucasian; 44 years; female; colon) cells were purchased from ATCC (Manassas, VA, USA). Caco-2 clone type CBBe1 (passage 24-25) and HT29-MTX cells (passage 70-73) were seeded separately using a Dulbecco´s modified Eagle´s medium (DMEM) with GlutaMAXTM and supplemented with 0.25 µg/mL amphotericin B, 1% (v/v) antibiotic-antimitotic mixture (final concentration of 100 U/mL penicillin and 100 µg/mL streptomycin), 1% (v/v) L-glutamine, 10% (v/v) inactive FBS, and 1% (v/v) non-essential amino acids obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). The cells were placed in an incubator at 37 °C under a 5% CO_2 environment and a water-saturated atmosphere (CellCulture® CO_2 Incubator, ESCO GB Ltd., Barnsley, UK). The culture medium was changed every two days until confluence.

3.10.2. MTT assay

The intestinal cells viability was assessed by the uptake and metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), an essential mitochondrial dye, by cell mitochondria as described by Pinto, Vieira, *et al.*[116]. *The assay was conducted on the SE at six different concentrations ranging between 31.25 and 1000* µg/mL. Three separate tests were conducted in triplicate for each concentration. In summary, cells were cultivated in a 96-well microplate at a density of 25×10^3 cells per milliliter of culture medium and incubated at 37 °C in a 5% CO_2 environment for 24 h. After that, the cells were incubated with different extract concentrations for 24 h at 37 °C in a 5% CO_2 atmosphere. The negative control was 1% (w/v) Triton X-100, while the positive control was DMEM. Following the removal of the extract, cells were washed with phosphate-buffered saline solution (PBS, pH 7.4), the MTT reagent dissolved in DMEM was added, and the microplate was incubated for 3 h at 37 °C to allow

the formation of formazan crystals. Upon the medium removal, 150 µL of DMSO was added to solubilize the blue formazan crystals, providing an estimate of the number of viable cells. The results were expressed as percentage (%) of cell viability and the absorbance was measured at 490 nm with background subtraction at 630 nm (Figure 13).

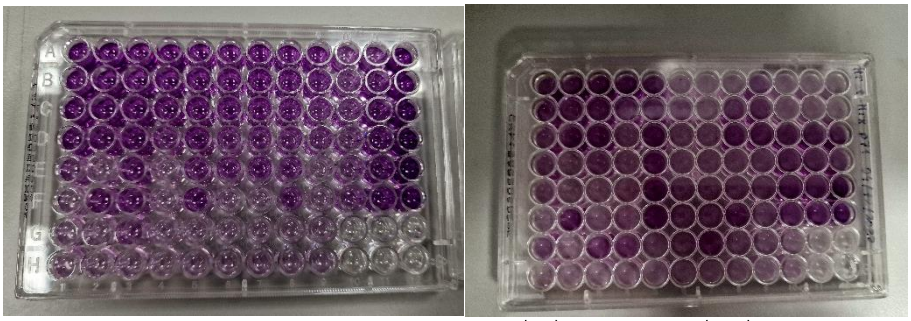

Figure 13 – Microplate of MTT assays on Caco-2 (left) and HT29-MTX (right) cells.

3.11. *In vitro* Intestinal Permeability Assay

The SE and SP digests obtained after the intestinal phase were exposed to a co-culture model composed of Caco-2 and HT29-MTX to simulate the intestinal permeability [117]. In tissue culture flasks (Orange Scientific, Belgium), Caco-2 and HT29-MTX cells were cultivated separately, as described above, and washed with HBSS after medium removal each 48 h. Using trypsin, cells were extracted when they were 90-95% confluent. Adhering to the co-culture model, a 90:10 ratio was applied to seed Caco-2 and HT29-MTX cells in the apical compartment (density of 1×10^5 cells/cm^2) of 12-well Transwell® plates (3 µm pore diameter, polycarbonate, 1.12 cm^2) and were cultivated at 37 °C under a 5% CO_2 environment for 21 days. The culture medium was changed every two days. On the 21st day, the culture medium was removed, cell monolayers were washed twice with HBSS, and the intestinal digests were added to the apical compartments. Aliquots (200 µL) were collected from the basolateral compartments at fixed times (0, 15, 30, 45, 60, 90, 120, 180, and 240 min), and the same volume of HBSS was added. HBSS was used as negative control, and the experiment was performed in triplicate. The intestinal co-culture model used is represented in Figure 14. To check the integrity of the cell monolayer, an EVOM epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA) with electrodes was employed to measure the transepithelial electrical resistance (TEER) during the cell culture and assay. The permeated samples were analyzed by LC/MS [117]. The results were expressed as the permeability percentage (%) of each phenolic compound, at each fixed time, across the intestinal barrier.

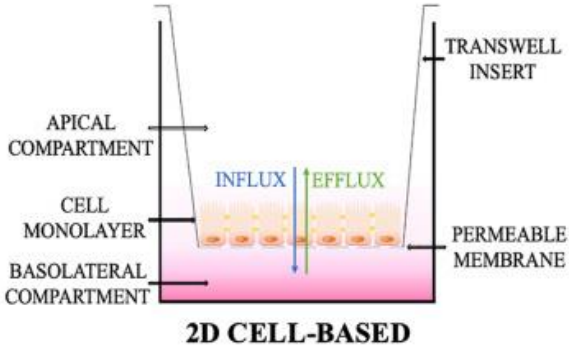

Figure 14 – *In vitro* intestinal permeability model scheme[118].

3.12. Phenolic profile by LC-MS

Liquid chromatography coupled with mass spectrometry (LC-MS) was used to assess the phytochemical profile of SP and SE and the respective digested fractions [119]. A 3.0 × 150 mm, 3.5 µm Agilent Eclipse XDB C-18 column was used for the stationary phase. The mobile phase was constituted by water containing 1% formic acid (A) and acetonitrile (B). The gradient starts at 90% A isocratic for 2 min; in 10 minutes, 25% A; and then in 15 minutes, 0% A. The column temperature was maintained at 30 °C, and the flow rate was adjusted to 0.4 mL/min. Two different MS platforms were used as detectors for qualitative purposes, including a quadrupolar ion trap (QTrap) working in negative ion mode and collecting data in the TDDS (transdermal drug delivery system) function. Compounds were identified based on their MS fragments and by comparison with the literature. For quantitative purposes, triple quadrupole MS 320 was used operating in negative mode. Transitions were obtained from the MS fragments observed in the ion trap and conditions for the triple quadrupole were optimized. The samples were filtered through 0.45 µm nylon filters and 20 µL were injected for the LC-MS measurements. MS spectra were recorded in negative ion mode within a *m/z* range of 150 to 2000. Based on the discovered compounds and reference standards that are available, reference compounds were selected. Results are expressed as mg of each compound per 100 g of dried sample (mg/100 g dw).

3.13. Statistical Analysis

Data were expressed as mean ± deviation of at least three independent experiments (n=3) carried out for each one of the three batches of extracts. The statistical analyses were performed using IBM SPSS

Statistics 27.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was employed followed by Tukey´s Honest Significant Difference (HSD) test with a significant level of $p < 0.05$.

4. Results and Discussion

4.1. Total Phenolic and Flavonoids Contents

The TPC and TFC of the undigested and digested samples are presented, respectively, in Tables 2 and 3.

Table 2– Total phenolic content obtained for *S. ramosissima* by-product and its extract before and after *in vitro* digestion.

Samples	*In vitro* simulated digestion					
	Oral digest		Gastric digest		Intestinal digest	
	TPC (µg GAE/100 mg dw)	Recovery (%)	TPC (µg GAE/100 mg dw)	Recovery (%)	TPC (µg GAE/100 mg dw)	Recovery (%)
SE	707.93 ± 3.99^{d,1}	37.41 ± 1.30	1053.72 ± 45.47^{c,1}	55.70 ± 2.73^{2}	2277.70 ± 83.80^{a,1}	120.38 ± 4.36^{2}
SP	233.99 ± 4.49^{d,2}	37.52 ± 0.81	524.44 ± 17.91^{c,2}	84.08 ± 2.97^{1}	1300.89 ± 35.17^{a,2}	208.57 ± 6.04^{1}
Undigested samples						
TPC (µg GAE/100 mg dw)						
Undigested SE	1892.25 ± 168.96 ^{b,1}		Undigested SP	623.76 ± 13.54 ^{b,2}		

GAE, gallic acid equivalents. dw, dry weight. SE, *S. ramosissima* by-product extract. SP, *S. ramosissima* by-product. Different letters (a, b, c, and d) indicate significant differences ($p < 0.05$) between undigested and digested samples for the same extract. Different numbers in superscript (1 and 2) indicate significant differences ($p < 0.05$) between SP and SE.

As shown in Table 2, the TPC was raised for both SE and SP samples during the *in vitro* digestion in the following order: oral < gastric < intestinal digests. However, the greatest value (2277.70 µg GAE/100 mg dw) was determined for the SE extract after the intestinal phase. Regarding the undigested samples, the highest value was obtained for the SE (1892.25 µg GAE/100 mg dw) which was expected since the extract contained a higher concentration of polyphenols when compared to the SP due to the limited solubility of polyphenols and their potential linkage to macromolecules (such as phenolic glycosides) or other nutrients (proteins, carbohydrates, and fatty acids) that can hinder the release of polyphenols [120]. At every stage of digestion, there were significant differences ($p < 0.05$) for SP and SE, as well as for all undigested and digested samples. The phenolic recovery also increased in the same order. Both SE and SP reached 37% of phenolic recovery after oral digestion, with non-significant differences ($p > 0.05$), increasing to 56% and 84%, respectively, after gastric digestion, demonstrating significant differences ($p < 0.05$). After intestinal digestion, the phenolic recovery reached 120% and 209%, respectively, for SE and SP, with significant differences between samples ($p < 0.05$). This indicates that the intestinal enzymes and pH conditions, such as pancreatin and bile salts, allowed a greater release of phenolic compounds from the SE and SP matrixes into the digestion medium, which is supported by other studies [121]. These results were also in line with Pinto *et al.* [122] who studied the effects of in vitro digestion in the phenolic concentration of chestnut shells.

Table 3 – Total flavonoid content obtained for *S. ramosissima* by-product and its extract before and after *in vitro* digestion.

Samples	*In vitro* simulated digestion					
	Oral digest		Gastric digest		Intestinal digest	
	TFC (µg CE/100 mg dw)	Recovery (%)	TFC (µg CE/100 mg dw)	Recovery (%)	TFC (µg CE/100 mg dw)	Recovery (%)
SE	328.39 ± 42.65^{b,1}	85.94 ± 10.93^{1}	386.08 ± 46.21^{1}	91.21 ± 10.92^{1}	410.19 ± 45.62	96.91 ± 10.78
SP	130.68 ± 19.41^{d,2}	31.42 ± 1.81^{2}	242.84 ± 37.29^{c,2}	58.32 ± 2.72^{2}	380.58 ± 29.14^{b}	92.37 ± 9.75

Undigested extracts			
TFC (µg CE/100 mg dw)			
Undigested SE	423.27 ± 25.62^{a}	Undigested SP	413.90 ± 66.02^{a}

CE, catechin equivalents. dw, dry weight. SE, *S. ramosissima* by-product extract. SP, *S. ramosissima* by-product. Different letters (a, b, c, and d) indicate significant differences ($p < 0.05$) between undigested and digested samples for the same extract. Different numbers in superscript (1, 2, and 3) indicate significant differences ($p < 0.05$) between SP and SE.

As can be observed in Table 3, the TFC outcomes were comparable to the TPC results, with concentrations increasing during digestion and reaching the highest results for the intestinal digests and the lowest for the oral digests. The highest TFC for the digested samples was obtained for the SE after the intestinal phase (410.19 µg *CE/100 mg dw).* In contrast to the oral samples, there were non-significant differences (*p > 0.05)* between the undigested and digested samples during the gastric and intestinal phases. Moreover, unlike the other phases, there were non-significant differences (*p > 0.05)* between SP and SE during the intestinal phase. Regarding the undigested samples, the SE sample achieved the highest value (423.27 µg CE/100 mg dw). The flavonoids recovery percentages increase following the same pattern. A more pronounced increase was observed for SP with recovery rates of 31%, 58%, and 92%, respectively, after oral, gastric, and intestinal digestions. Although the SE achieved identical flavonoids bioaccessibility upon intestinal digestion (96%), higher recovery rates were determined after oral and gastric phases (86% and 91%, respectively) when compared to SP. No significant differences (*p > 0.05)* were observed between SE and SP samples for the intestinal phase regarding bioaccessibility percentage. These results were identical to the ones obtained for TPC, suggesting that flavonoids are more readily retrieved in the intestinal environment due to the action of pancreatin and neutral pH [123].

In general, the phenolic compounds is higher than the concentration of flavonoids in *Salicornia* plants, which is in line with some studies [124, 125] that attested TPC and TFC variations during in vitro gastrointestinal digestion and indicated that the digestion phase determines the concentration of phenolic and flavonoid compounds. This is primarily due to the low stability of phenolic compounds when exposed to digestive enzymes and pH changes that promote their breakdown and biotransformation into

simpler molecules. As evidenced by these authors, the phenolic and flavonoid concentrations also increased during digestion, while the highest amounts of these molecules were determined in the undigested samples.

4.2. Impact of *in vitro* digestion on the antioxidant/antiradical properties

The effects of *in vitro* simulated digestion on the antiradical and antioxidant properties of *S. ramosissima* by-product were also studied. The results for ABTS, DPPH, and FRAP assays are summarized in Table 4.

Table 4 – Antioxidants/antiradical properties of *S. ramosissima* by-product and its extract before and after digestion evaluated by FRAP, ABTS, and DPPH assays.

Samples	FRAP	ABTS	DPPH
	µg FSE/100 mg dw	µg AAE/100 mg dw	µg TE/100 mg dw
SE Oral	1422.89 ± 145.43^{c,1}	835.35 ± 56.95^{d,1}	336.57 ± 51.61^{c}
SE Gastric	2105.09 ± 259.80^{b,1}	914.43 ± 82.88^{c,1}	421.20 ± 31.60^{b}
SE Intestinal	2594.28 ± 222.37^{1}	4828.16 ± 190.97^{a,1}	220.12 ± 31.95^{d}
Undigested SE	2990.61 ± 463.80^{a}	1959.08 ± 97.36^{b}	1351.10 ± 228.23^{a}
SP Oral	615.50 ± 32.45^{c,2}	425.76 ± 22.02^{d,2}	156.70 ± 21.09^{d}
SP Gastric	1194.64 ± 120.16^{b,2}	643.89 ± 52.60^{c,2}	272.69 ± 38.58^{b}
SP Intestinal	1817.02 ± 176.64^{2}	1313.40 ± 54.45^{a,2}	193.24 ± 26.74^{c}
Undigested SP	1813.89 ± 177.73^{a}	762.36 ± 110.96^{b}	467.90 ± 53.70^{a}

Values are expressed as mean ± standard deviation. FRAP, ferric reducing antioxidant power. ABTS, 2,2-azino-bis-3 ethylbenzothiazoline-6-sulphonic acid. DPPH, 2,2'- diphenyl-1-picrylhydrazyl. dw, dry weight. SE, *S. ramosissima* by-product extract. SP, *S. ramosissima* by-product. Different letters (a, b, c, and d) indicate significant differences ($p < 0.05$) between undigested and digested samples for the same extract. Different numbers in superscript (1, 2, and 3) indicate significant differences ($p < 0.05$) between SP and SE.

The results of the FRAP assay indicate that the undigested SE had the highest antioxidant activity (2990.61 µg FSE/100 mg dw), followed by the SE intestinal digest (2594.22 µg FSE/100 mg dw). The antioxidant activity of the SP also rose during the digestion process, reaching the maximum value after the intestinal digestion (1817.02 µg FSE/100 mg dw), which was similar to the undigested SP (1813.89 µg FSE/100 mg dw). Additionally, the data demonstrated that there were significant differences ($p < 0.05$) between SP and SE for every phase, except for a non-significant difference between the intestinal and the undigested samples for both SE and SP ($p > 0.05$).

Likewise, there was a considerable improvement in the antiradical activity of SE and SP measured by ABTS assay throughout digestion. The SE intestinal digest (4828.16 µg AAE/100 mg dw) was the best

ABTS scavenger, followed by the undigested SE (1959.08µg AAE/100 mg dw). For the SP, lower results were attained when compared to SE, with the highest value being determined after the intestinal phase (1313.40 µg AAE/100 mg dw), followed by the undigested SP (762.36 µg AAE/100 mg dw). For every phase, there were significant differences ($p < 0.05$) between SP and SE samples, as well as between the undigested and digested samples for the same extract.

In what concerns to the DPPH assay, a distinct pattern was observed, with the highest values being obtained for the undigested SE and SP samples (1351.10 µg TE/100 mg dw and 467.90 µg TE/100 mg dw, respectively). The DPPH scavenging response increased from the oral to gastric phases but dropped by half in the intestinal phase for both samples. According to Rice-Evans *et al.* [126], the observed results can be attributed to the fact that the chemical structures of phenolics enhance their capacity to scavenge free radicals. The racemization of molecules is known to be influenced by pH, and it most likely results in the creation of two chiral enantiomers with distinct biological reactivities [127]. Antioxidant molecules may, therefore, be more reactive during digestion, especially at an acidic pH (gastric medium) as opposed to a neutral one (intestinal medium). Also, non-significant differences ($p > 0.05$) were observed between all phases for SP and SE samples, but significant differences ($p < 0.05$) occurred between digested and undigested samples for both SP and SE.

Overall, regarding SE samples, the antioxidant/antiradical properties improved during the digestion process for FRAP and ABTS assays, highlighting better antioxidant/antiradical responses for the undigested fraction. However, concerning the ABTS assay, the highest value was obtained after the intestinal phase rather than the undigested fraction. Regarding SP samples, the antioxidant/antiradical properties also improved during the digestion process for FRAP and ABTS assays, despite better antioxidant responses being determined for the intestinal fraction. In the DPPH assay, there was a decrease in the antiradical activity of the intestinal digests for both samples, being the best responses obtained for the undigested fraction.

4.3. Scavenging Activity Against Reactive Oxygen Species

The results of the scavenging ability against pro-oxidant reactive species of both undigested and digested SP and SE are shown in Table 5.

Table 5 – Scavenging potential of undigested and digested *S. ramosissima* by-product and its extract against ROS.

Samples	Reactive oxygen species						
	$O_2^{\cdot-}$		HOCl		H_2O_2		ROO$^{\cdot}$
	IC_{50} (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)	Inhibition (%)	µg TE/mg dw
SE Oral	nd	99.95 ± 0.05	nd	48.71 ± 2.82^{a,1}	nd	65.49 ± 4.32^{b}	1.18 ± 0.07^{d}
SE Gastric	nd	100.19 ± 0.04	nd	19.43 ± 0.17	nd	52.67 ± 5.63	13.12 ± 0.49^{c}
SE Intestinal	nd	90.95 ± 9.20^{1}	nd	33.50 ± 3.36^{b}	nd	79.74 ± 1.59^{a,1}	32.14 ± 0.93^{b,1}
Undigested SE	85.45 ± 1.62	nd	10.59 ± 0.47^{c}	nd	47.02 ± 0.26^{c}	nd	38.03 ± 0.53^{a}
SP Oral	nd	98.38 ± 0.09^{a}	nd	32.16 ± 0.95^{2}	nd	nd	0.60 ± 0.08
SP Gastric	nd	90.74 ± 0.10^{b}	nd	27.56 ± 0.16	nd	nd	12.14 ± 1.70^{b}
SP Intestinal	nd	52.78 ± 2.42^{2}	nd	31.28 ± 3.85	nd	63.98 ± 0.51^{a,2}	12.33 ± 2.03^{a,2}
Undigested SP	nd	37.76 ± 4. 06^{c}	37.63 ± 0.87	nd	nd	42.72 ± 2.55^{b}	4.41 ± 0.29^{c}
Positive control							
Gallic acid	23.84 ± 0.32	nd	3.67 ± 0.04	nd	400.71 ± 5.68	nd	nd

Values are expressed as mean ± standard error of the mean. dw, dry weight. SE, *S. ramosissima* by-product extract. SP, *S. ramosissima* by-product. TE, Trolox equivalents. Different letters (a, b, c, and d) indicate significant differences ($p < 0.05$) between undigested and digested samples for the same extract. Different numbers in superscript (1,2, and 3) indicate significant differences ($p < 0.05$) between SP and SE. nd, not determined.

Regarding the $O_2^{\cdot-}$ assay, identical inhibitory rates were achieved for the three SE digests, as well as for oral and gastric digests of SP, with inhibition percentages being higher than 90%, without significant differences ($p > 0.05$). Otherwise, SP intestinal digest was the least efficient in capturing $O_2^{\cdot-}$ among digests, with 53% inhibition. A possible explanation for this result can be due to the quenching power of the polyphenols recovered after the intestinal digestion of SP, being weaker and not able to transfer its protons to the $O_2^{\cdot-}$ that can be neutralized. For the undigested samples, the SE presented a much stronger quenching power than SP, being required 85.45 µg/mL to neutralize 50% of the $O_2^{\cdot-}$ produced in the tested medium. Out of all samples, the undigested SP had the lowest value with 38% inhibition, which was expected since the sample was examined in its unprocessed state. Significant differences ($p < 0.05$) were also observed between the intestinal phase of SP and SP samples, as well as between the digested and undigested samples of SP, except for the intestinal phase.

The HOCl quenching capacity increased in the following order: gastric < intestinal < oral digests < undigested samples. The digested samples showed the lowest scavenging potential against this reactive species, with the SE oral digest exhibiting the highest value (49%). Nonetheless, regarding the undigested samples, the most effective scavenger was SE, exhibiting the lowest IC_{50} value (10.59

µg/mL), followed by SP (IC_{50} = 37.63 µg/mL). Significant differences ($p < 0.05$) were observed between SE and SP oral phase samples, as well as between the digested and undigested samples from SE oral and intestinal.

Considering the H_2O_2 assay, the scavenging potential of digested and undigested samples varied widely. Regarding SE, the H_2O_2 counteracting ability improved in the following order: gastric < oral < intestinal digests < undigested sample. The SE intestinal digest has the highest scavenging ability (79% inhibition) among the digests and showed significant differences ($p < 0.05$) from the undigested samples. This also occurred in the oral phase. Both SP oral and gastric digests demonstrated no activity against this reactive species, while the intestinal digest displayed 64% inhibition, possibly as a result of the formation of polyphenols in the intestinal medium capable of scavenging H_2O_2 due to its ability to donate hydrogen. Within the undigested samples, the best scavenger was SE (IC_{50} = 47.02 µg/mL), while SP only inhibited 43% of the H_2O_2 produced in the tested medium. There were also significant differences ($p < 0.05$) between SP and SE intestinal samples.

The ORAC assay was employed to assess the $ROO^{\cdot}$ counteracting ability by estimating the protective benefits against LPO damage. In this experiment, the $ROO^{\cdot}$ scavenging potential increased during the digestion phases for both SP and SE samples, being the highest value obtained for the SE intestinal digest (32.14 µg TE/mg dw), with significant differences ($p < 0.05$) from the SP intestinal sample. Among the undigested samples, SE showed a higher $ROO^{\cdot}$ quenching capacity (38.03 µg TE/mg dw) than SP (4.41 µg TE/mg dw). Except for SP oral, there were significant differences ($p < 0.05$) between all the undigested and digested samples for both SP and SE.

Overall, the results of the ROS scavenging efficiency highlighted the potential contribution of phenolic composition to the bioactivity of digested and undigested *S. ramosissima* by-product, which have shown effective radicals counteracting effects in recent studies [68, 69].

4.4. Enzymatic Assays

The inhibitory capacity of undigested and digested SP and SE on AChE and antioxidant enzymes activities was assessed and the results are shown in Table 6.

Table 6 – Enzymatic assays of undigested and digested *S. ramosissima* by-product and its extract.

Samples	Enzymatic Assays				
	AChE	SOD	CAT	GSH-Px	LPO
	Inhibition (%)		CAT Activity (nmol/min/g dw)	GSH-Px Activity (U/g dw)	MDA Concentration (nmol MDA/g dw)
SE Oral	33.61 ± 3.41^{a}	68.84 ± 1.37^{b,1}	37.41 ± 5.84^{c,1}	46.17 ± 0.91^{c}	174.07 ± 6.89
SE Gastric	20.41 ± 0.13	80.44 ± 0.34^{a,1}	186.84 ± 18.28^{a,1}	81.32 ± 7.54^{b}	338.85 ± 12.47
SE Intestinal	21.55 ± 0.92^{2}	25.85 ± 3.76^{d,2}	43.48 ± 2.05^{b,2}	124.75 ± 27.77	2163.90 ± 103.20^{1}
Undigested SE	16.92 ± 0.23^{b}	40.10 ± 4.78^{c}	30.04 ± 3.75^{d}	186.34 ± 59.33^{a}	nd
SP Oral	34.67 ± 2.63	39.61 ± 4.10^{2}	18.78 ± 0.00^{2}	7.96 ± 1.07	194.73 ± 7.75^{c}
SP Gastric	24.58 ± 1.96^{b}	65.34 ± 1.20^{a,2}	41.15 ± 6.64^{b,2}	22.11 ± 3.14	550.76 ± 36.09
SP Intestinal	31.18 ± 0.86^{1}	57.49 ± 0.34^{b,1}	52.89 ± 8.48^{a,1}	53.20 ± 7.77	1637.63 ± 107.58^{a,2}
Undigested SP	33.08 ± 3.66^{a}	32.97 ± 0.17^{c}	30.02 ± 0.56^{c}	18.01 ± 4.27	480.21 ± 11.20^{b}

Values are expressed as mean ± standard error of the mean. dw, dry weight. SE, *S. ramosissima* by-product extract. SP, *S. ramosissima* by-product. TE, Trolox equivalents. Different letters (a, b, c, and d) indicate significant differences ($p < 0.05$) between undigested and digested samples for the same extract. Different numbers in superscript (1,2, and 3) indicate significant differences ($p < 0.05$) between SP and SE. n.d., not determined.

AChEs are enzymes that hydrolyze the neurotransmitter acetylcholine to acetate and choline. The inhibition of these enzymes can be useful as a therapeutic target for neurodegenerative disorders through the restoration of acetylcholine levels in the brain [128]. The complete blockage of this enzyme is lethal [129]. In terms of the AChE inhibition, the undigested SE yielded the lowest result (17% inhibition). Significant differences ($p < 0.05$) were observed between the SP and SE samples in comparison to the intestinal phases. Additionally, substantial variations ($p < 0.05$) were identified between the undigested SP and the SP gastric phase, as well as between the undigested SE and the SE oral phase. Nevertheless, SE reached the highest AChE inhibition after oral digestion (34%), while similar results were obtained after gastric and intestinal digestion (20% and 22% inhibition, respectively). Regarding SP, the undigested sample and its oral and intestinal digests showed identical AChE inhibitory potential (33%, 35%, and 31%, respectively), while lower enzymatic inhibitory capacity was attained after gastric digestion (25% inhibition). These results are consistent with TPC and TFC results, indicating that the neuroprotective potential of *S. ramosissima* upon digestion can be related to the slow release of these bioactive molecules that raise the acetylcholine levels.

SOD is one of the most important antioxidant enzymes, catalyzing the dismutation of the superoxide anion (O_2^-) into H_2O_2 and O_2. A compromise in SOD activity can have broad ramifications in cell metabolism, ranging from defects in amino acid metabolism to increases in oxidative damage of

deoxyribonucleic acid (DNA) molecule. [130]. Therefore, the inhibition of this enzyme can lead to oxidative stress [131]. The highest inhibition percentages were determined after gastric digestion for both SE and SP samples (80% and 65% inhibition, respectively). Concerning SE, the lowest result was obtained upon intestinal digestion (25%), indicating that the intestinal samples had a lower inhibition percentage, as would be predicted given the increased concentration of polyphenolic compounds. The drop of SOD inhibition through digestion emphasizes the advantages of *Salicornia´s* bioactive composition. In terms of significant differences, there was just one non-significant difference ($p > 0.05$) between the SP oral sample and the undigested one.

CAT is an enzyme present in most aerobic cells, being enrolled in the detoxification of H_2O_2 by catalyzing its decomposition into molecular oxygen and water without the production of free radicals, helping against oxidative stress [132, 133]. Concerning SE, the highest value was observed for the gastric phase (186.84 nmol/min/g dw), while the lowest was achieved with the oral phase of the SP sample (18.78 nmol/min/g dw). Additionally, when compared to the undigested samples, the digested ones yield better findings, demonstrating the benefits of including this plant in the diet. Regarding statistical differences, the results were identical to the SOD assay.

GSH-Px is an intracellular enzyme responsible for the detoxification of peroxides in living cells, playing a crucial role in protecting cells from damage by free radicals formed by peroxide decomposition. GSH-Px reduces the peroxides to alcohols using glutathione and, consequently, prevents the formation of free radicals [134]. Regarding SE, the highest value was attested for the undigested sample (186.34 U/g dw), while the lowest was achieved for the oral phase of the SP sample (7.96 U/g dw). As was already anticipated, the digested samples showed an increase in these enzyme´s activity during digestion, supporting the earlier findings. Between undigested and digested SP samples, as well as between SP and SE samples, there were no significant differences ($p > 0.05$). Nonetheless, these results showed significant differences ($p < 0.05$) between undigested SE and digested SE oral and gastric samples.

LPO consists of the degradation of lipids caused by oxidative damage, and it is a useful marker of oxidative stress, contributing to the pathology of many diseases, such as diabetes and Alzheimer's disease [135]. An organism's lipid peroxidation process is triggered by free radicals. One of the end products of polyunsaturated fatty acid peroxidation in cells is malondialdehyde (MDA). Its overproduction is caused by a rise in free radicals [136]. The highest results were attested for the intestinal phases of SE and SP samples (216.90 and 1637.63 nmol MDA/g dw), with significant differences ($p < 0.05$) among them and between undigested and digested SP oral and intestinal samples. These values support the advantages of consuming this plant since it lowers the impact of oxidative stress.

Based on the results obtained it is possible to conclude that *S. ramosissima* by-product is very beneficial for human health even after gastrointestinal digestion, reducing oxidative stress, stimulating antioxidant enzymes activities, improving neurological function, and therefore, preventing many diseases. All these benefits may be possible by incorporating this plant alongside diet.

4.5. MTT assay

An accurate and sensitive way to measure the cellular metabolic activity is through the MTT assay that indicates cell viability after exposure to a compound or extract. The MTT assay is based on the reduction of yellow water-soluble tetrazolium dye (MTT) to purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase [137]. After being dissolved in DMSO, the formazan product is examined spectrophotometrically, and the comparison of the spectra of cells treated and untreated with the compound or extract provides an estimate of the cytotoxicity degree [138]. Caco-2 and HT29-MTX cell lines are frequently employed in cell viability assays due to their unique characteristics and physiological relevance, particularly in studies related to the gastrointestinal tract. Caco-2 cells, derived from human colorectal adenocarcinoma, are widely recognized for their ability to differentiate into a monolayer that mimics the enterocytes of the small intestine when cultured under specific conditions. These cells develop features such as microvilli, tight junctions, and active transport systems, closely resembling the intestinal epithelial barrier. This makes Caco-2 cells an invaluable model for investigating oral drug absorption, nutrient transport, and interactions with the intestinal lining [139]. HT29-MTX cells are a subline of the HT29 human colorectal adenocarcinoma cell line, selected for their ability to produce mucus. These cells secrete mucin, the primary component of mucus, and are employed to mimic the mucus-secreting goblet cells of the intestinal epithelium. The production of a protective mucus layer by HT29-MTX cells is crucial for studies focusing on the interactions of various substances with the mucus barrier, as well as the overall barrier properties of the intestinal epithelium [140]. The use of these cell lines in tandem allows for a detailed assessment of the absorptive and protective functions of the intestinal barrier, thereby enhancing the physiological relevance of *in vitro* studies [141]. Figure 15 shows the results obtained after SE extract exposure to both cell lines.

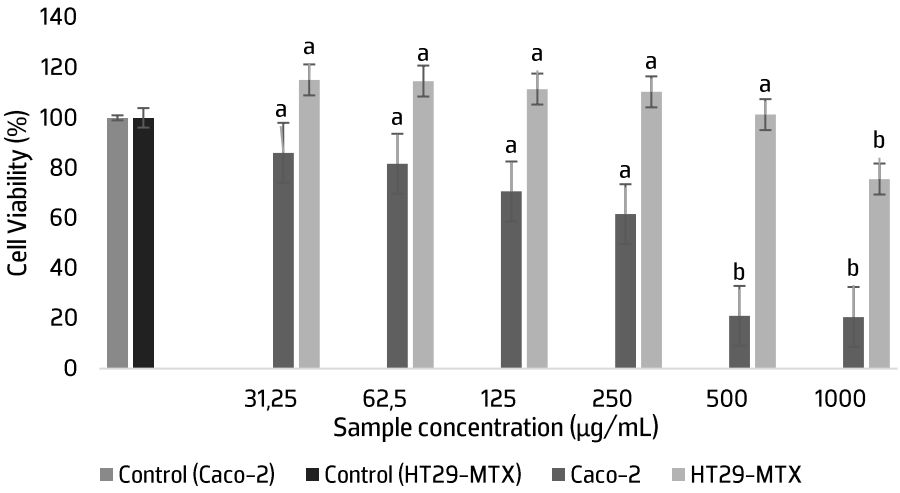

Figure 15 – Effects of *S. ramosissima* by-product extract (SE) on the viability of Caco-2 and HT29-MTX cells at a range of concentrations between 31.25 and 1000 µg/mL.

As shown in Figure 15, it is noteworthy to conclude that the highest Caco-2 and HT29-MTX viability was achieved after exposure to the concentration of 31.25 µg/mL, displaying a viability higher than 80% for both cell lines. Regarding Caco-2 cell line, it is possible to conclude that the extract is safe for this cell line at concentrations up to 250 µg/mL since the cell viability remained above 60%. However, a significant viability reduction was observed after exposure to concentrations of 500 and 1000 µg/mL. On the other hand, the HT29-MTX viability still reached 76% after exposure to the highest SE tested concentration (1000 µg/mL), being classified as safe for concentrations up to 1000 µg/mL. These findings are consistent with Silva *et al.* [36] who reported no discernible reduction in HT29-MTX viability after exposure to *S. ramosissima* extracts prepared by MAE and CE up to 1000 µg/mL. Furthermore, in comparison, there was generally lower viability in both cell lines for the highest SE concentrations tested, while the viability percentages were higher for HT29-MTX cells than for Caco-2 cells.

4.6. Phenolic profile by LC-MS

Phenolic compounds are transformed through metabolic processes that influence their accessibility, absorption, and action in the human body. Therefore, *in vitro* and *in vivo* studies have been developed to analyze the metabolic routes of phenolic compounds during digestion and how the resulting metabolites affect their activity in promoting human health [142]. Although *in vivo* studies offer a realistic view of phenolic metabolism, they are significantly impacted by individual differences, challenges in sample preparation and analytical techniques, as well as potential interactions with other nutrients [142, 143].

Given the biological significance, this study aimed to elucidate the true bioactivity of phenolic compounds from *S. ramosissima* upon human consumption. It focused on understanding the effects of digestive enzymes and pH variations at different digestion stages on the degradation and/or metabolization of phenolic compounds, and consequently, on their bioaccessibility, using an *in vitro* digestion model.

Figure 16 illustrates the chromatograms of the undigested SE, while Figure 17 represents the chromatograms of the SE oral, gastric, and intestinal digests. Table 7 summarizes the phenolic compounds present in undigested SE and SP.

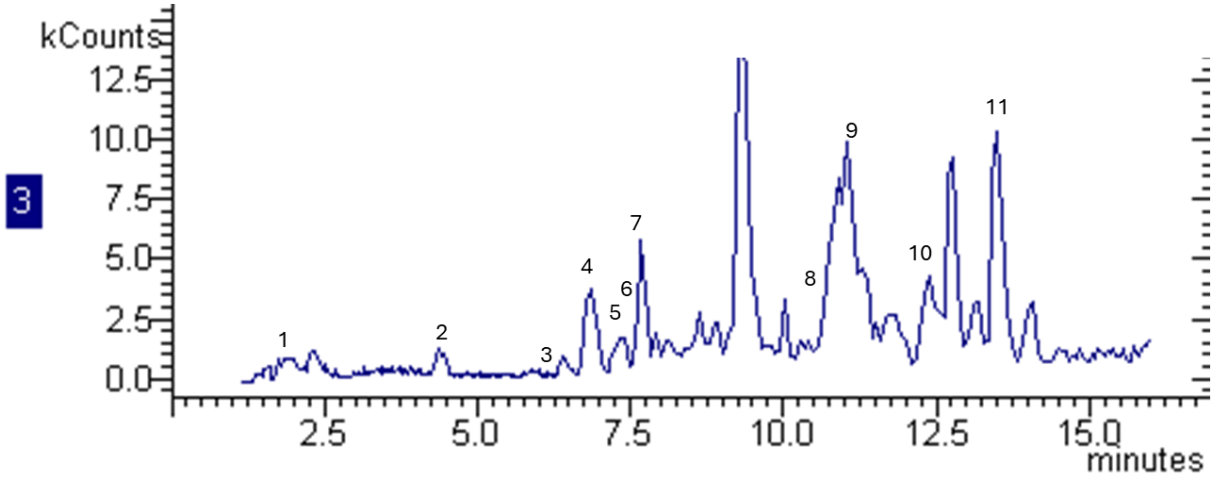

Figure 16 – LC–MS chromatograms of undigested SE extract. Peak identification id as follows: (1) Quinic acid; (2) 3-CQA; (3) 5-CQA; (4) Quinic acid sulphate; (5) 4-CQA; (6) 3-FQA; (7) Gallocatechin; (8) 3,4-diCQA; (9) 3,5-diCQA; (10) 4,5-diCQA; and (11) Chikusetsusapoinin IV A/ calenduloside G isomer.

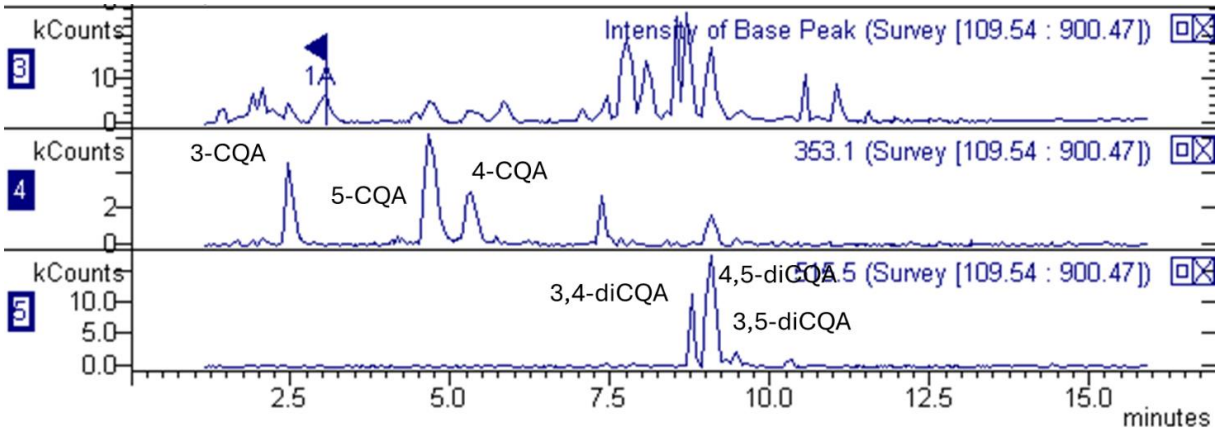

Figure 17– LC-MS chromatogram of the SE extract after oral (3), gastric (4), and intestinal (5) phases.

Table 7 – *S. ramosissima* SE-SP constituents identified by LC-MS.

SE-SP extracts constituents				
Nº	Rt (min)	$[M-H]^-$	Fragments	Compounds
1	1.8	191	111.1	Quinic acid
2	4.3	353.1	191.1 179.1	3- caffeoylquinic acid
3	6.7	353.1	191.1	5- caffeoylquinic acid
4	7.26	271.1	191.1	Quinic acid sulphate
5	7.3	353.1	173.1 191	Cryptochlorogenic acid
6	7.34	367.2	193.1	3-feruloylquinic acid
7	7.5	305.2	225	Gallocatechin
8	8.3	515.1	353.1 335 173	3,4-dicaffeoylquinic acid
9	8.75	515.1	353.2 191.5 179	3,5-dicaffeoylquinic acid
10	8.82	515.1	353 191 179	4,5- dicaffeoylquinic acid
11	10.9	793.2	673 631 613 569 455	Chikusetsusapoinin IV A/ calenduloside G isomer
12	11.5	809.2	647 471	Hex + HexUA + hederagenin
13	12.4	809.2	647 471	Hex + HexUA +hederagenin
14	13.5	793.2	673 631 613 569 455	Chikusetsusapoinin IV A/ calenduloside G isomer
15	14.2	823.2	673 631 613 569 455	Acetylsaikosaponin D or isomer
16	15.2	823.2	673 631 613 569 455	Acetylsaikosaponin D or isomer
17		777.1	615.3921,539.3361,469.330,43 7.3059 ,407.2939	23-Hydroxy-13 β,28 β -epoxyolean-11-ene-16-one3-*O*- β -D-glucopyranosyl-(1→3)- β -D-fucopyranoside

The chromatogram reveals a peak with a molecular ion $[M-H]^-$ at *m/z* 191, identified as quinic acid through comparison with a reference compound. Three isobaric species, corresponding to intense peaks with a UV max at 330 nm, were identified as the isomers 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA). Additionally, cryptochlorogenic acid (4-CQA) was characterized by an MS2 spectrum with a base peak at *m/z* 191 and a minor fragment at *m/z* 179, which is in agreement with the literature [144, 145]. Another derivative, 3-FQA (3-O-feruloylquinic acid), was identified by a molecular ion $[M-H]^-$ at *m/z* 367 and an intense fragment at *m/z* 191, corresponding to quinic acid [145].

Three other peaks, showing a UV max at 330 nm and a molecular ion $[M-H]^-$ at *m/z* 515 in negative ion mode, were attributed to three isomers of dicaffeoylquinic acid derivatives: 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA [145]. Minor peaks at *m/z* 353 and 367 indicated the presence of caffeoylquinic acid and feruloylquinic acid [144]. Chlorogenic acids (CQAs) offer numerous advantages due to their diverse biological activities such as antioxidant properties, being capable of scavenging free radicals and reducing oxidative stress, protecting cells from damage, and lowering the risk of chronic diseases such as cancer and cardiovascular diseases [146]. Moreover, the anti-inflammatory and antimicrobial effects make them a potential candidate for use in food preservation and as therapeutic agents [147-149].

At longer retention times, some peaks with insignificant UV absorption were detected via mass spectrometry, suggesting the presence of saponins in *Salicornia* [150]. Peaks with a molecular ion [M-

H]$^-$ at *m/z* 793, consistent with chikusetsusapoinin IV A (also known as calenduloside G), were formed by an oleanolic acid aglycone, one hexose, and one hexuronic acid moiety [151].

Two isomeric saponins with a molecular ion [M-H] $^-$ at *m/z* 809 were tentatively identified as hederagenin derivatives linked with hexose and hexuronic acid [152]. Additionally, a peak with a molecular ion [M-H]$^-$ at *m/z* 823 was attributed to Acetylsaikosaponin D [151]. These compounds are known for their antioxidant properties [153, 154], hepatoprotective [155], and cholesterol-lowering effects [156]. Similar compositions were observed for SP, with varying constituent amounts.

Regarding digestion, the initial analysis of the SE extract showed no significant compositional changes after oral digestion. However, the gastric digestion phase was marked by notable alterations in the chromatographic profile, with the most prominent change being an increase in the intensity of peaks corresponding to monocaffeoylquinic acids and a relative decrease in dicaffeoylquinic derivatives. This suggests the partial cleavage of the ester linkages, resulting in the formation of mono CQA from the identified diCQA. Regarding saponins, no significant changes were observed, probably due to the greater resistance of their ether linkages.

The SE extract, after oral and intestinal *in vitro* digestion, displayed several peaks with significant absorption in the diode array, characterized by typical UV maxima at 330 nm, indicative of hydroxycinnamic acids, or at 280 nm, suggesting the presence of flavan-3-ols. These findings highlight changes in the phenolic composition of the *Salicornia* extract during *in vitro* digestion, indicating the impact of the digestive enzymes and pH on the phenolic compound's retention in each phase. Thus, SE is richer in polyphenols when compared to SP. Both extracts also contain saponins. The *in vitro* digestion does not alter the saponin profile but affects caffeoylquinic acids. DiCQA are partially hydrolyzed to CQA, which is particularly evident in SP, where CQA peaks are quite intense after digestion, being almost undetectable before digestion.

4.7. *In vitro* Intestinal Permeability Assay

Polyphenols and their metabolites are absorbed through the digestive tract after metabolism. Several *in vitro* cell models, including the Caco-2/HT29-MTX co-culture model, have been developed as promising tools to mimic the human intestinal epithelium and measure the phenolic permeation in light of the dynamic and intricate nature of the intestinal absorption process [157]. A frequently utilized quantitative method to assess the integrity of tight junction dynamics in cell culture models of epithelial monolayers is called transepithelial electrical resistance (TEER) [158]. The SE and SP digested fractions obtained after the intestinal phase were tested on an intestinal co-culture model. The TEER values measured during the 21 days of cultivation and 4 h of assay are presented, respectively, in Figures 18 and 19.

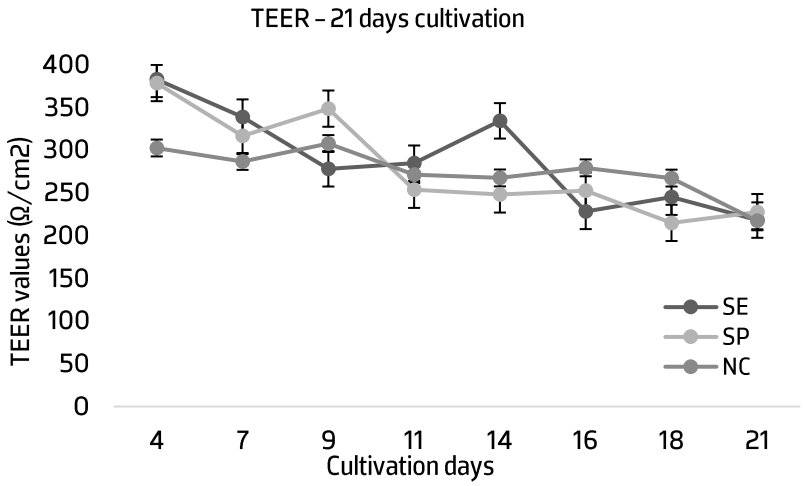

Figure 18 – TEER values during 21 days of the co-culture model cultivation.

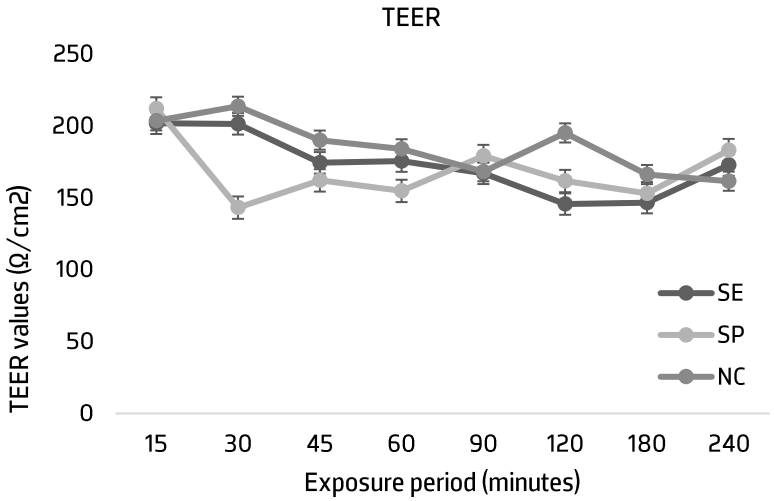

Figure 19 – TEER values during the intestinal permeability assay.

As depicted in Figure 18, the TEER values regarding SE decreased until day 9 (277.98 Ω/cm²), then increased until day 14 (334.08 Ω/cm²), and stabilized until day 21 (218.04 Ω/cm²). Regarding SP, the

TEER values decreased until day 11 (253.47 Ω/cm²) and remained constant until day 21 (227.30 Ω/cm²), suggesting a stable cell growth rate. During the 240 min assay, the TEER values remained constant for SE and SP (Figure 19), ranging between 145.50 (120 minutes) and 172.58 (240 minutes) for SE and from 152.99 (180 minutes) to 183.00 (240 minutes) for SP. These results ensured the integrity of the cell barrier during the permeability assay.

For the intestinal permeability assay, intestinal digests of SP and SE samples were evaluated using a Caco-2/HT29-MTX co-culture model, a well-established system for mimicking the human intestinal environment. Tables 8 and 9 summarize the permeation rates of the different phenolic compounds at each time point for both SE and SP intestinal digests, respectively.

Table 8 – Permeation of phenolic compounds and metabolites from SE intestinal digest across the intestinal co-culture model.

	SE							
Compounds	15 min	30 min	45 min	60 min	90 min	120 min	180 min	240 min
Quinic acid	0	0	0	1.4±0.11	2.23±0.11^{b}	3±0.15^{c}	3.7±0.15^{f}	5.11±0.26^{e}
3- caffeoylquinic acid	0	0	0	0	0	2.93±0.15^{d}	4.14±0.21^{e}	5.86±0.29^{d}
5- caffeoylquinic acid	0	0	0	0	0	20.28±1.01	9.98±0.5^{a}	31.93±1.6
Cryptochlorogenic acid	0	0	0	0	0	17.5±0.88	77.9±3.9	34.84±1.74
3-feruloylquinic acid	0	0	0	0	0	0	0	0
Gallocatechin	0	0	0	0	1.17±0.06^{d}	1.89±0.1^{e}	3.35±0.17^{g}	4±0.20^{f}
3,4-dicaffeoylquinic acid	0	0	0	0	2.28±0.12^{a}	7.88±0039^{a}	9.65±0.49^{b}	14.01±0.7^{a}
3,5- dicaffeoylquinic acid	0	0	0	0	2.04±0.1	7.04±0.35^{b}	8.63±0.43^{c}	12.52±0.63^{b}
4,5- dicaffeoylquinic acid	0	0	0	0	2.04±0.1^{c}	7.04±0.35^{b}	8.63±0.43^{c}	12.52±0.63^{b}
Chikusetsusapoinin IV A/ calenduloside G isomer	0.01±0.00	0.01±0.00	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{h}	0.02±0.00^{h}	0.02±0.00^{j}	3.09±0.15
Hex + HexUA + hederagenin	0	0	0.22±0.02	0.24±0.02	1.14±0.06^{e}	5.39±0.27	7.5±0.38^{d}	8.8±0.44
Hex + HexUA +hederagenin	0	0	0	0	0	0	0	0
Chikusetsusapoinin IV A/ calenduloside G isomer	0.02±0.00	0.02±0.00	0.03±0.01^{a}	0.03±0.00^{a}	0.03±0.01^{g}	0.04±0.00^{g}	0.05±0.01^{i}	6.6±0.33^{c}
Acetylsaikosaponin D or isomer	0	0	0.7±0.04	1.06±0.05	2.06±0.1	3.52±0.18	6.67±0.33	7.21±0.36
Acetylsaikosaponin D or isomer	0	0	0	0	0	0	0	0
23-Hydroxy-13 β,28 β - epoxyolean-1	0	0	0	0	1.11±0.06^{f}	1.71±0.09^{f}	2.71±0.14^{h}	3.45±0.17^{g}

Values are expressed as mean ± standard error of the mean. SE, *S. ramosissima* by-product extract. Different letters (a-j) indicate significant differences between the various compounds identified, at the same time.

Table 9 – Permeation of phenolic compounds and metabolites from SP intestinal digest across the intestinal co-culture model.

	SP							
Compounds	15 min	30 min	45 min	60 min	90 min	120 min	180 min	240 min
Quinic acid	0	2.61±0.13	4.04±0.2	6.74±0.34^{a}	8.09±0.41^{a}	14.84±0.74^{a}	22.27±1.11	25.98±1.3^{b}
3- caffeoylquinic acid	0	0	0	0	0	1.67±0.09^{h}	6.95±0.35^{d}	12.11±0.61^{d}
5- caffeoylquinic acid	0	0	0	0	0	2.24±0.12^{f}	3.8±0.19^{g}	4.88±0.25^{i}
Cryptochlorogenic acid	0	0	0	0	0	1±0.05^{i}	4.45±0.23^{e}	8.5±0.43^{g}
Gallocatechin	0	0	0	0	0.33±0.02^{j}	0.52±0.03^{k}	0.93±0.05^{i}	1.11±0.06^{k}
3,4- dicaffeoylquinic acid	0	0	0	0	1.79±0.09^{g}	9.23±0.47^{d}	12.55±0.63	16.43±0.82
3,5- dicaffeoylquinic acid	0	0	0	0	7.88±0.4^{c}	9.65±0.49^{e}	15.42±0.77	22.16±1.11
4,5- dicaffeoylquinic acid	0	0	0	0	7.94±0.4^{b}	13.82±0.69^{b}	18.63±0.93	26.73±1.34^{a}
Chikusetsusapoinin IV A/ calenduloside G isomer	29.14±1.46	4.86±0.25	5.67±0.29	6.61±0.33^{b}	7.71±0.39^{d}	9±0.45^{c}	10.5±0.53^{a}	12.39±0.62^{c}
Hex + HexUA + hederagenin	0	0	0	0.02±0.01^{c}	0.09±0.01^{l}	0.31±0.02^{l}	0.46±0.03^{k}	0.69±0.04^{m}
Hex + HexUA +hederagenin	0	0	0.02±0.00	0.02±0.01^{c}	0.11±0.01^{k}	0.54±0.03^{j}	0.75±0.04^{j}	0.88±0.04^{l}
Chikusetsusapoinin IV A/ calenduloside G isomer	0	0	0	0	0	0	0	0.14±0.01^{n}
Acetylsaikosaponin D or isomer	0.45±0.03^{b}	0.9±0.05^{a}	1.43±0.08^{a}	2.86±0.15	4.05±0.21^{e}	6.94±0.35	9.15±0.46^{b}	11.57±0.58^{e}
Acetylsaikosaponin D or isomer	0.47±0.02^{a}	0.7±0.04^{b}	1.18±0.06^{b}	1.97±0.10	3.88±0.20^{f}	4.67±0.24	7.57±0.38^{c}	9.92±0.50^{f}
23-Hydroxy-13 β,28 β -epoxyolean-1	0	0	0	0	1.11±0.06^{h}	1.71±0.09^{g}	2.71±0.14^{h}	3.45±0.17^{j}
Oleic Acid	0	0	0	0	1.1±0.06^{i}	3.11±0.16^{e}	3.81±0.19^{f}	5.51±0.28^{h}

Values are expressed as mean ± standard error of the mean. SP, *S. ramosissima* by-product. Different letters (a-n) indicate significant differences between the various compounds identified, for the same time.

The principal phenolic compounds that permeated across the intestinal barrier were 5 CQAs (3-FQA only present in SE) and 3 metabolites, 1 flavonoid, 1 triterpenoid, 2 triterpenoid saponins, 1 saponins derivative and oleic acid (only present in SP).

Considering the SE samples, the permeation of bioactive compounds enhanced in the following order: Chikusetsusapoinin IV A < 23-Hydroxy-13 β,28 β -epoxyolean-1 < Gallocatechin < Quinic acid < 3-CQA < Chikusetsusapoinin IV A < Acetylsaikosaponin D < Hex + HexUA + hederagenin < 3,5-diCQA = 4,5-diCQA < 3,4-diCQA < 5-CQA < 4-CQA with increasing rates from 15 to 240 min. 4-CQA achieved the maximum intestinal permeability (34.84%) at 240 min, followed by 5-CQA (31.93% at 240 min). 23-hydroxy-13 β, 28 β -epoxyolean-1 achieved 3.45% permeation, followed by chikusetsusapoinin IV A (Rt=10.9) with the lowest permeation (3.09%).

Regarding the SP samples, the phenolics permeation improved as follows: Chikusetsusapoinin IV A < Hex + HexUA + hederagenin < Hex + HexUA + hederagenin < 23-Hydroxy-13 β,28 β -epoxyolean-1<5-CQA < Oleic Acid < 4-CQA < Acetylsaikosaponin D < 3-CQA < Chikusetsusapoinin IV A < 3,4-diCQA < 3,5-diCQA < Quinic acid < 4,5-diCQA. 4.5 diCQA achieved the maximum intestinal permeability of 26.73% at 240

min, followed by Quinic acid (25.98% at 240 min). Chikusetsusapoinin IV A (Rt=10.9) and Hex + HexUA + hederagenin (Rt=11.5) had the lowest permeation, respectively, 0.14 % and 0.69%. In general, significant differences (p > 0.05) were observed across all time points when comparing the various compounds. Moreover, a substantial proportion of the phenolic compounds, characterized by limited intestinal permeability, are likely retained within the intestinal barrier [159]. This retention can be attributed to their complex chemical structures and the biotransformation processes that convert these compounds into metabolites do not detect. This difficulty is further exacerbated by the absence of appropriate standards and potential interferences from cellular constituents, which may not allow the translocation of these compounds from the apical to the basolateral side of the intestinal epithelium [159]. Despite this, phenolic compounds that remain in the cell barrier might still provide health benefits, such as cytoprotective effects, which could help to mitigate the oxidative stress associated with the transport of phenolic compounds across cell membranes [160].

At longer retention times, some peaks with insignificant UV absorption were detected via mass spectrometry, suggesting the presence of saponins in *Salicornia* [150]. Peaks with a molecular ion $[M-H]^-$ at m/z 793, consistent with Chikusetsusapoinin IV A (also known as calenduloside G), were formed by an oleanolic acid aglycone, one hexose, and one hexuronic acid moiety.[151]

Two isomeric saponins with a molecular ion $[M-H]^-$ at m/z 809 were tentatively identified as hederagenin derivatives linked with hexose and hexuronic acid [152]. Additionally, a peak with a molecular ion $[M-H]^-$ at m/z 823 was attributed to Acetylsaikosaponin D [151]. These compounds are known by their antioxidant properties [153, 154], hepatoprotective [155] and cholesterol-lowering effects [156]. Similar compositions were observed for SP, with varying constituent amounts.

5. Future perspectives

The future research on the valorization of *S. ramosissima* by-product holds significant potential, particularly in enhancing their application within the nutraceutical and food industries. One of the key avenues for further investigation involves the conduction of animal studies to understand the *in vivo* effects, being crucial to evaluate the efficacy, safety, and potential therapeutic benefits of this plant.

Another promising research area is the application of encapsulation techniques to improve the stability of polyphenols derived from *S. ramosissima* by-product. By enhancing the stability of these compounds, encapsulation can facilitate a targeted delivery to specific tissues, thereby maximizing the therapeutic effects. This approach could improve the bioavailability of polyphenols and ensure their sustained release, which is vital for achieving the desired health outcomes [161].

In addition to encapsulation, exploring other green extraction techniques for the isolation of polyphenols will be interesting. These environmentally friendly methods can increase the yield and purity of the extracted compounds, making the process more sustainable and cost-effective [14]. Implementing these advanced extraction techniques will contribute to a more efficient use of *S. ramosissima* by-product, enhancing their overall value.

Finally, it is crucial to identify the most suitable functional food matrix for the incorporation of *Salicornia* by-product. This involves characterizing the nutritional and phytochemical composition of the selected food, as well as assessing its biological properties[161]. By doing so, the full potential of *S. ramosissima* by-product can be realized, paving the way for their successful integration into the nutraceutical and food industries, where they can offer considerable health benefits and contribute to sustainable food production practices.

6. Conclusion

This study aimed to valorize *S. ramosissima* by-products by investigating the effects of the *in vitro* gastrointestinal digestion and intestinal permeability on the bioaccessibility and bioactivity of bioactive compounds. The research focuses on understanding how these digestive processes impact the bioavailability and potential health benefits of phenolics, contributing to the sustainable use of this by-product in food and pharmaceutical applications.

The results obtained highlighted the dynamic changes in phenolic and flavonoid content in *S. ramosissima* through *in vitro* gastrointestinal digestion. Phenolic compounds were found in higher concentrations than flavonoids, and the bioactivity of these compounds increased during digestion. The antioxidant and antiradical properties, measured by FRAP and ABTS assays, improved through digestion, with notable differences between the undigested and intestinal phases, especially for SE samples. However, a reduction in the antiradical activity was observed in the DPPH assay for intestinal digests, with the undigested fraction showing the highest activity.

In the $O_2^{\cdot-}$ assay, SE samples exhibited stronger quenching abilities than SP ones, though the efficiency dropped for the SP intestinal digest, possibly due to reduced proton transference capacity. Additionally, SE samples showed better inhibition in the HOCl and H_2O_2 assays, with a strong scavenging capacity demonstrated across phases, especially after intestinal digestion.

The ORAC assay attested the increasing $ROO^{\cdot}$ scavenging potential during digestion for SE and SP, with the SE intestinal digest showing the highest activity. AChE inhibition results were higher for SE after oral digestion, with significant differences observed between the undigested and digested phases.

Notably, the cell viability assays indicated that SE extracts were safe for Caco-2 and HT29-MTX cells at concentrations up to 1000 µg/mL, with a higher viability obtained for HT29-MTX cells. The TEER values remained stable during the permeability assays, indicating a good epithelial barrier integrity during the experiments. The phenolic permeability varied, with 4-CQA showing the highest permeation among SE compounds and 4,5-diCQA for the intestinal digestion ones.

These findings emphasize the significant antioxidant, cytoprotective, and neuroprotective potential of *S. ramosissima* by-products, especially after gastrointestinal digestion, supporting their incorporation in dietary products to combat oxidative stress and promote gut and brain health. One limitation of the present study was the inability to fully account for the biotransformation of phenolic compounds during digestion, which may have affected the detection of certain metabolites.

Overall, this research contributes to understanding how gastrointestinal processes affect the bioactivity of phenolic compounds from *S. ramosissima*, offering promising insights for their use in enhancing human health.

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P.PORTO

ESCOLA SUPERIOR DE SAÚDE

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MESTRADO

BIOQUÍMICA EM SAÚDE