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Antioxidant properties and enzymatic activity towards acetylcholinesterase in the macroalga *Fucus vesiculosus* and its variation with sex, growth stage and seasonality

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Abstract

Fucus vesiculosus has been a subject of study due to being an extremely common coastal seaweed and to the benefic properties associated with it, either as a result of its high polyphenol content - in particular its phlorotannins, present only in brown algae – and the antioxidant activity associated with it. Additionally, new studies explore *F. vesiculosus* usage to inhibit acetylcholinesterase activity (AChE), an enzyme that catalyzes the breakdown of acetylcholine (ACh), associated with the treatment of symptons related to Alzheimer's Disease. The high mineral content of *F. vesiculosus* is also of a great interest, both due to its nutritional importance in human health, and to detect the presence of heavy metals in the alga's constitution, as it can clear its usage for human consumption and also study *F. vesiculosus* potential as a bioaccumulator and bioremediator.

In this thesis, a characterization of *F. vesiculosus* provinient of Tagus estuary was carried out, particularly to study the influence of the growth stage and gender on different aspects of this seaweed properties: total phenolic content (TPC); antioxidant activity; compounds detected through high-performance liquid chromatography with diode array detector (HPLC-DAD); inhibition towards acetylcholinesterase and mineral composition of *F. vesiculosus.*

Four groups indicative of the growth stage of *F. vesiculosus* were defined according to the thallus length (thallus <20 cm; 20-25 cm; 25-30 cm; >30 cm) and the initial sample were divided amongst these. Thereafter, these four groups were divided by their gender (male or female) following microscopic observation, totaling eight groups. Finally, all samples were extracted at two different temperatures (100 ºC and 25 ºC), resulting in sixteen different groups of analyzed samples.

Results suggest that the males belonging to the youngest growth stage (thallus <20 cm) demonstrate the higher phenolic content, across the two types of extract. Furthermore, the antioxidant activity assay shows a similar tendency. This may be due to phlorotannins remaining in shorter oligomeric forms when the thallus is younger, and such forms are associated with higher antioxidant activities, thus corroborating this data. *F. vesiculosus* compound analysis using HPLC-DAD suggests variations in components throughout its development, that may indicate that certain compounds are important in specifics parts of *F. vesiculosus* growth. On the other hand, out of the sixteen sets of samples, only three show some inhibition towards acetylcholinesterase (20-25 cm males, 25-30 cm males and >30 cm females) as the samples were not purified before this assay and thus the presence of fucoidans and other polysaccharides hinders the inhibitory activity. Lastly, elemental analysis further demonstrates the high nutritional value that *F. vesiculosus* minerals provide and shows no bioaccumulation of the heavy metals studied.

As such, this thesis provides additional insight in the beneficial properties provided by *F. vesiculosus*, while providing a different approach in this alga's characterization. By studying the influence of the growth stage and gender on the aspects described, it is possible to suggest an ideal harvesting time for *F. vesiculosus* from the Tagus estuary if one intends to fully optimize the alga's phenolic content and antioxidant properties: the youngest male specimens (thallus length <20 cm).

Keywords: *Fucus vesiculosus,* phlorotannin, antioxidant activity, acetylcholinesterase, heavy metals

Resumo

Ao longo dos anos, produtos baseados em plantas têm sido cada vez mais reconhecidos pelo seu valor intrínseco em vários setores benéficos na saúde humana, seja pelo contributo nutricional que fornecem, na medicina, como suplementos ou alternativas a produtos sintéticos. As algas são um grupo particularmente de interesse, cujas propriedades que as caracterizam já estão ligadas ao seu extenso uso tradicional em diversas regiões, como no Este Asiático (Japão, China, Tailândia, por exemplo). São organismos que residem em zonas costeiras, normalmente associados a um substrato rochoso, e que podem ser encontrados em inúmeras regiões ao longo do globo, sendo que são geralmente divididos de acordo com a pigmentação em três divisões: Rhodophyta (algas vermelhas), Phaeophyta (algas castanhas) e Chlorophyta (algas verdes).

Em Portugal, *Fucus* vesiculosus, também conhecido como bodelha, é uma das espécies mais comuns encontradas na costa lisboeta. Classificada dentro da divisão Phaepohyta, a *F. vesiculosus*, possui um conjunto variado de características que a tornam bastante versátil na sua utilização, particularmente o seu teor baixo em gorduras, elevado conteúdo mineral e valor alto de polifenóis e polissacáridos não-digeríveis. Para além disto, *F. vesiculosus* contém florotaninos na sua estrutura, um subtipo de polifenóis derivados de floroglucinol característicos de algas castanhas e que estão normalmente associados a uma maior atividade antioxidante. Este tipo de propriedade é extremamente procurado devido aos seus benefícios na saúde humana – por norma, no processo de respiração aeróbica, são formadas espécies reativas de oxigénio (ROS) e espécies reativas de nitrogénio (RNS) onde as mais perigosas são o anião superóxido (O_2^-) e radical hidroxilo (OH). Estes radicais livres são mantidos em controlo pelo mecanismo de defesa de antioxidantes do organismo, de maneira a evitar os efeitos adversos que provêm da sobreprodução de ROS e RNS. Deste modo, um sistema debilitado de antioxidantes está associado a um aumento de ROS no organismo, o que conduz a stress oxidativo no organismo. Como consequência, este tipo de stress é relacionado com vários tipo de doenças crónicas e degenerativas, como: (i) cancro; (ii) doenças cardiovasculares, como aterosclerose, isquemia, hipertensão ou cardiomiopatia; (iii) doenças neurológicas, como Alzheimer, Parkinson, esclerose múltipla e esclerose lateral amiotrófica; (iv) doenças pulmonares, como asma e doença pulmonar obstrutiva crônica; (v) artrite reumatóide; (vi) doenças renais (glomerulonefrite e nefrite túbulo-intersticial); (vii) doenças oculares (cataratas) e (viii) pré-eclâmpsia em medicina pré-natal.

Como brevemente descrito, uma das aplicações descritas de *F. vesiculosus* é no tratamento da doença de Alzheimer. A doença de Alzheimer é um tipo de doença cerebral que danifica neurónios envolvidos em funções cognitivas. Os estados iniciais desta doença normalmente não são imediatamente detetados, sendo que os seus sintomas apenas começam a ser reconhecidos anos mais tarde, associados a sinais normais de envelhecimento. Sendo uma doença neurodegenerativa, a doença de Alzheimer progride ao longo dos anos, sendo que os sintomas iniciais podem impedir o normal funcionamento de certas atividades planeadas, evoluindo para a maneira de pensar, aprender, e, em estados mais avançados, afetar funções básicas do organismo, sendo que os pacientes nesta fase requerem cuidado por parte de terceiros. Os tratamentos atuais não impedem nem diminuem o dano dos neurónios, pelo que o tipo de medicina atualmente existente, procura atenuar os sintomas da doença. É nesta perspetiva que a utilização de *F. vesiculosus* se insere no contexto da doença de Alzheimer. No decorrer desta, por norma, existe perca de função colinérgica no sistema nervoso central, que contribui para a degradação de funções cognitivas associadas à doença de Alzheimer, o que resulta numa deficiência em acetilcolina (ACh), uma molécula que medeia neurotransmissão no cérebro. Assim, um tipo de tratamento sintomático associado à doença de Alzheimer consiste na inibição da enzima que cataliza a degradação da ACh – a acetilcolinesterase (AChE).

Devido ao habitat onde se inserem (estuário do Tejo) as amostras de *F. vesiculosus* utilizadas neste estudo, o perfil de elementos presentes nesta alga é também de grande importância. Por um lado, fornece informações mais específicas relativamente aos minerais mais comuns e importantes nutricionalmente de *F. vesiculosus* (como Na, K, I, Ca, Fe, Mg, Cu, Zn e Mn). Por outro lado, permite o estudo do conteúdo de metais pesados que possam ter sido acumulados pelas amostras recolhidas no estuário do Tejo, não só permitindo a análise do potencial desta amostra na inclusão no mercado, como também o potencial desta alga como bioindicadora, bioremediadora ou bioacumuladora no habitat onde se insere.

O objetivo desta dissertação passou por efetuar uma caracterização de *F. vesiculosus* colhida no estuário do Tejo. Tendo em conta a variação que esta alga pode sofrer na sua constituição, de acordo com o habitat onde cresce, condições ambientais e maturação, estudou-se a influência do estado de crescimento e sexo de *F. vesiculosus* em vários aspetos desta alga: conteúdo fenólico total (TPC); atividade antioxidante e a sua relação com TPC; compostos detetados por cromatografia líquida de alta eficiência com detetor de fotodíodos (HPLC-DAD) ao longo do crescimento e por sexo; inibição da acetilcolinesterase e composição mineral de *F. vesiculosus.* Para o efeito, foram definidos quatro grupos que indicam o estado de crescimento da alga, agrupados de acordo com o comprimento do talo (talo <20; 20-25 cm; 25-30 cm; >30 cm). Posteriormente, estes grupos foram subdivididos por sexo (macho ou fêmea) após observação de cada amostra à lupa e ao microscópio, obtendo quatro grupos diferentes de estado de crescimento por sexo (oito no total). Por último, as amostras foram extraídas a duas temperaturas de extração diferentes (25 ºC e 100 ºC), totalizando 16 grupos de amostras analisadas.

Relativamente ao teor total de fenóis, os resultados sugerem que os machos no estado de crescimento mais jovem (talo <20 cm) possuem um teor fenólico mais elevado, partilhado nos dois tipos de extrações efetuadas. Isto poderá ser explicado pela presença de florotaninos de menor peso molecular nas fases mais jovens de crescimento, onde são mais comuns. Estes florotaninos de menor peso molecular são associados com uma atividade antioxidante maior, algo que também é corroborado pelos dados obtidos, que demonstram uma tendência similar entre maior atividade antioxidante e teor fenólico total nos machos mais jovens. No entanto, é importante referir que, por norma, os estados de crescimento mais altos (talo >30 cm) alcançaram valores altos em teor fenólicos, mas não mostraram a mesma tendência descrita para os estados mais jovens relativamente à atividade antioxidante.

Na análise efetuada por RD- HPLC-DAD, os resultados sugerem que existe variação de componentes ao longo dos diversos estados de crescimento estudados, o que pode implicar a existência mais reforçada de certos compostos durante o desenvolvimento de *F. vesiculosus* e são, desta forma, encontrados em maior concentração. No entanto, futuramente, será necessária a identificação destes compostos através de cromatografia líquida – espetrometria de massa (LC-MS-MS) para melhor avaliar a variação detetada neste estudo.

Das amostras analisadas de *F. vesiculosus*, apenas três (20-25 cm machos, 25-30 cm machos e >30 cm fêmeas) mostrar alguma inibição da acetilcolinesterase. Estes resultados poderão ser explicados devido às amostras não terem sido purificadas previamente ao ensaio. Para além disto, a presença de fucoidanos e outros polissacáridos, que não possuem atividade inibitória poderão ter influenciado os resultados. Deste modo, no futuro, purificação das amostras utilizando extração em fase sólida (SPE) previamente ao ensaio pode originar extratos com maior atividade inibitória.

Por último, a analise de elementos efetuada comprovou o potencial nutricional que *F. vesiculosus* fornece enquanto suplemento alimentar, especificamente pelo valor do iodo no tratamento do hipertiroidismo. Adicionalmente, os metais pesados detetados não ultrapassaram os limites superiores estabelecidos pelas organizações responsáveis pelas suas normas. No entanto, neste estudo, cádmio, um metal pesado anteriormente estudado em *F.vesiculosus* por ser facilmente bioacumulado por esta espécie,

não foi estudado nesta dissertação, pelo que trabalhos futuros com amostras provenientes do estuário do Tejo deverão também incluir este elemento na sua caracterização.

Em suma, esta dissertação provou vários dos benefícios providenciados por *F. vesiculosus*, para além de fornecer diretrizes no seu tempo ideal de colheita de acordo com o aspeto desejável que se procura, particularmente para o teor fenólico e atividade antioxidante, onde os estados mais jovens machos demonstraram ser de maior valor.

Palavras-chave: *Fucus vesiculosus*, florotanino, atividade antioxidante, acetilcolinesterase, metais pesados

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List of Abbreviations

AD: Alzheimer's Disease

ACh: Acetylcholine

AChE: Acetylcholinesterase

AChI: Acetylcholine Iodide

BChE: Butyrylcholinesterase

B.W: Body weight

CVD: Cardiovascular Diseases

DAD: Diode Array Detector

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid)

DW: Dry Weight

EFSA: European Food Safety Authority

FAO: Food and Agriculture Organization

HPLC: High-Performance Liquid Chromatography

JECFA: Joint FAO/WHO Expert Committee on Food Additives

PTWI: Provisional Tolerable Weekly Intake

OBIS: Ocean Biogeographic Information System

RDI: Recommended Daily Intake

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

SPE: Solid Phase Extraction

TPC: Total Phenolic Content

TRXF: Total Reflection X-Ray Fluorescence Spectrometry

UL: Upper Intake Level

WHO: World Health Organization

1. Introduction

1.1. Plant-based products are in growing demand

Historically, either directly or indirectly, plants have always been used for their value in human health, nutrition or therapy. Vegetarian based diets (pesco-vegetarian, ovo-lacto-vegetarian or vegan) are also gaining a growing interest, as an increasingly larger number of people seek alternatives to a meat-rich diet[1]. Although these are some more general types of vegetarian diets, they also may vary broadly between individuals, according to each one needs and restrictions[2]. Plant-based diets have been evidenced to reduce the risk of certain chronic diseases, for example cardiovascular disease (CVD), certain types of cancer and type-2 diabetes[2]. Albeit these factors, also associated with the lifestyles practiced by the population who follow vegetarian diets, restricting food consumption to certain food or food groups will not provide all the necessary nutrients that the human body needs, and even though this is true for all types of diets, dietary practices should aim to provide the suggested nutrient intakes[3].

On the other hand, plant-based products have also shown a growing demand as an alternative to synthetic products. Although traditionally, in countries like China, Egypt and Sumer, where the medicinal and therapeutic effects of the local flora have always been known since early centuries and are intertwined with the cultures of these regions, in recent years there has been an increasing interest in plant-based products and alternative therapeutic treatments[4]. An emerging alternative use for this type of natural products is its application in various cancer treatments, as many plant-based compounds have less toxic effects than current treatments, such as chemotherapy. Plant secondary metabolites, particularly polyphenols, barassinosteroids and taxols have been identified for their anticancer properties[5]

1.2. Seaweeds and their beneficial effects

A good subset of natural products known widely for their valuable health properties for humans are seaweed. Found largely in coastal zones throughout various regions in the world, their effects and usage are tied with the countries they are found in. In Japan, for example, use and consumption of various seaweed species, such as *Laminaria, Undaria, Prophyra* and *Gelidium* is reported as early as AD 701[6].

Algae are, in a broad sense, oxygen-generating, photosynthetic organisms other than land plant and lichens[7], being heterogenous aggregations of organisms belonging to a broad range of evolutionary lineages, and are very diverse from a genetic standpoint[8]. Therefore, taxonomically, algae do not have a fixed distribution, and their classification has changed many times over the last few decades. Although around 40000 species have been identified, many others remain unknown, as algae species estimate reaching over a million. Different phycologists suggest various divisions, ranging between 5 and 16, mainly due to the difficulty of distributing microalgae[9].

As briefly described, marine macroalgae are organisms similar to plants that reside in coastal areas, normally attached to a rocky substrate. They are generally classified according to their pigmentation and are distributed by three divisions: Rhodophyta (red), Phaeophyta (brown) and Chrolophyta (green)[10]. Besides serving as a food source in regions that traditionally consume them, they have a tremendous varied number of applications, either in human health or the environment. In human health, it has been reported that macroalgae can be used as an antibiotic[11], antiviral[12,13] and antifungal[14]; Kahalide F, a marine-derived cyclic depsipeptide, found that Bryopsis sp. has also shown a potent cytotoxic activity, inhibiting cell growth and multiplication in human cancer cell lines and is currently being investigated as a potential treatment to certain types of cancer, particularly, lung, prostate and breast cancer[15]; many brown, green and red algae have been described to have anti-inflammatory[16,17,18] and antioxidant[19,20] properties. From a biological point of view, macroalgae serve a major role in bioremediation as they often absorb certain elements. This is especially important in the control of high concentrations of heavy metal, and other toxic pathogens in estuaries and regions alike, for example cadmium[21,22]. Additionally, they can also be used in the treatment of wastewater, as they can remove nutrients such as nitrogen and phosphorous, and they offer an alternative to the use of chemicals in this process, as commercial technologies are already available in the market[23]. Another very interesting application of algae is its potential in providing biofuel as an alternative to fossil fuels, which currently is very sought after. In regions with a large coastline, where seaweed is widely harvested, biomass from seaweed can be used to synthetize biofuels. In large-scale road and air transports, where the energy demands are very high, alternative solutions to fossil fuels can contribute largely to meeting climate-change targets[24]. Although some methods regarding the production of biofuels, for example, biohydrogen via dark fermentation and biomethane via anaerobic digestion, have already been showcased[25], an increase in the yield of biofuel synthesis is still needed. Excluding processes of macroalgae cultivation, which is also an issue of its own in some species, several processes within biofuel production, particularly pre-treatments with higher efficiency, polysaccharide degradation, co-utilization of hexose and pentose need to be addressed in order to scale up the biofuel manufacturing[26]. In coming years, as processing technology evolves, this area of macroalgae applications should be followed with great interest. Lastly, macroalgae can also be applied in areas such as cosmetics, in the production of oils, scrubs, shampoos or algal soaps, for example, due to lipids present in their constitution[27].

1.3. Biology, distribution and reproduction of *Fucus vesiculosus*

Fucus, besides being important ecologically and economically, it is also one the most abundant species in the North Atlantic and temperate coastal areas[28]. As such, and because of its consistent seasonality in reproduction, it serves as a good research subject regarding *Fucus vesiculosus* properties and applications. Furthermore, due to its consistency in reproduction seasonality, despite its variations previously mentioned, the properties of the species can also be studied comparatively, particularly between genders and growth stage, as will be described in detail. Due to its high presence in the Tagus Estuary, *F. vesiculosus* was chosen as the *Fucus* species to study in this work.

F. vesiculosus, also known as bladderwrack, is a common marine alga that is found in coastal areas, generally in temperate and cold zones of the Pacific and Atlantic Oceans, being a very common fucoid and dominant in the mid-intertidal on rocky shores on both sides of the North Atlantic[29]. It is also found in a broad range of wave exposures and tolerates a wide range of salinities. Its distribution is displayed in Figure 1.1.

Figure 1.1. – Worldwide and European distribution of *F. vesiculosus*. Each dot represents an occurrence, in approximate 27088 records retrieved from the Ocean Biogeographic Information System (OBIS)[30–91].

According to the data collected, in Portugal, most of *F. vesiculosus* is found on the central to northern open coast, and southern estuaries like the Tagus River estuary, where the samples for this study were collected. *F. vesiculosus* is a perennial seaweed, meaning they are able to survive and remain healthy through several years and grow new branches every year, from the base. These new branches are called fronds which grow in successive years and given the large length some of these fronds can achieve it may suggest that *Fucus* species may attain longer lifespans. A study by Carballeira et. al[92] performed in Baltic and Galicia populations of *F. vesiculosus* reports that one pair of pneumatocysts (air bladders) is produced at the base of each new dichotomies. In addition to the fronds length, this data was used to try to determine the age of thalli in this particular study.

Regarding its life cycle, *F. vesiculosus* follows a similar pattern to other Fucales: the free-living plant is diploid, and the gametes go through meiosis during their formation[93]. When reproductive maturity is achieved, the tips of the vegetative branches mature into receptacles, that contain conceptacles where the female and male gametes (oogonia and antheridia, respectively) are formed within[94]. The oogonia go through meiosis and mitosis to produce eight egg cells whereas the antheridia produce several haploid sperm. Once maturity is achieved, the outer wall of the oogonium ruptures and the eggs are released from the conceptacle. The inner walls then disassociate to free the eggs and can be fertilized, although sperm are able to penetrate the oogonial sac before egg release (Figure 1.2)[94].

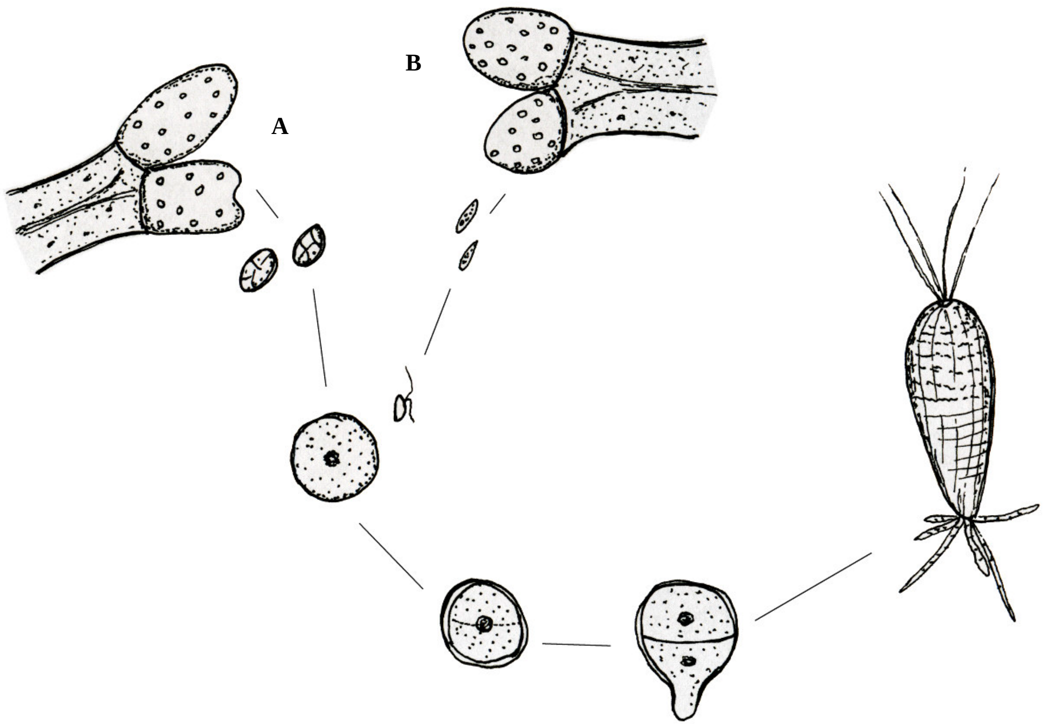

Figure 1.2. – Life stages and reproductive cycle of *F. vesiculosus*[94]. A and B represent the alga female and male receptacles, respectively (during its diploid stage, which is the free-living stage of *F. vesiculosus*, being the haploid stage reserved to the gametes – thus, the sexual life cycle is gametic). The gametes are produced in these structures, being released after maturation, where fertilization happens externally. The fertilized egg then produces an adhesive that is used to attach to the substrate. The egg then polarizes according to the light direction and is further attached by the cell developed in the first cell division, a rhizoid cell, and eventually develops into a germling.

F. vesiculosus reproduction is generally marked by its seasonality, where many abiotic factors may be used as environmental cues to induce reproduction or meet the requirements for this process[95]. In this group of seaweeds, these kinds of environmental factors not only serve to induce reproduction, but also influence the duration and its period, with photoperiod being the most common factor influencing reproduction[96]. Short-day conditions usually influence the formation of receptacles, whereas in long-day conditions the apices remain in their vegetative state [97]. The increase in temperature, on the other hand, either by itself or in combination with solar radiation, induce receptacle and gamete formation[98]. Lastly, the release of gametes appears to be regulated by circadian or circalunar cycles, which additionally may be associated with environmental factors, such as water salinity, tidal waves and height[99]. Following these experiments, *F. vesiculosus* populations generally seem to release gametes either in early summer (May - July), in the autumn (September – October), or in both seasons. Receptacle maturation is reached during the preceding autumn (under short-day conditions) if their gametes are released in the summer and maturing in the spring. On the other hand, individuals that release their gametes during the autumn season, initiate their receptacle maturation in the previous spring, maturating over the summer and initiating the release in September – October. In both cases, after gamete release, decaying receptacles are abscised and the development of new vegetative tips is initiated before the next reproductive cycle. This way, a full reproductive cycle in *F. vesiculosus* encompasses a duration of approximately 6-9 month[95].

The differentiation between male and female specimens, which was of particular interest in the present study, is done by observation of cross sections of the conceptacles of the samples through hand lenses and/or under a microscope. Oogonia are quite large and can be easily distinguished from male gametangia with a hand lens, but microscopic observation is necessary to identify the antheridiophores, tree-like structures that contain antheridia on their terminal tips of the branches - these undergo meiosis to form the male gametes, antherozoids (Figure 1.3). As such, it is required to carefully observe these structures as to differentiate male conceptacles from female ones that have not yet matured, as they cannot be distinguished at low magnification using a hand lens[100].

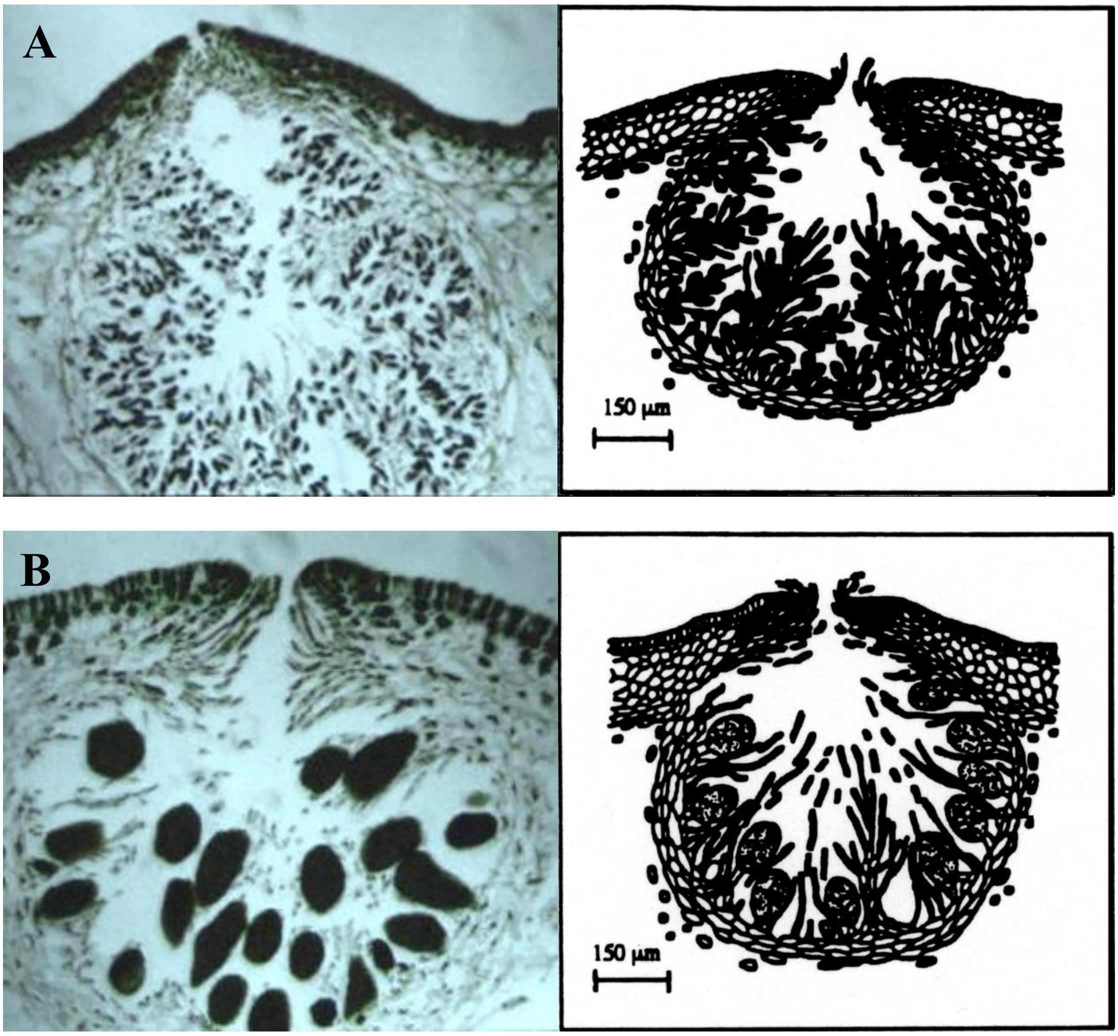

Figure 1.3. – Representation of male conceptacle (A) – containing the male gametes, antheridia, the small, dark tree-like structures – and female conceptacle (B) – containing the female gametes, oogonia, the large and bulb-shaped structures represented.

1.4. *F. vesiculosus* – bioactive compounds

As with other seaweeds, *F. vesiculosus* composition varies a lot according to its habitat, environmental conditions and maturity[101]. In general, although bladderwrack presents a high protein content relative to other brown algae species[101], it is considered to have a low protein content, in accordance with most brown seaweeds industrially exploited. Although variable throughout the year, *F. vesiculosus* also shows a low-fat content[102]. In regard to its mineral composition, the high ash level constitutes a good premise, as in seaweed, high content of ash is linked to higher level of minerals[103]. Furthermore, the high contents of polyphenols and non-digestible polysaccharides - dietary fiber – also provide *F. vesiculosus* beneficial health effects in its consumption[104]. While polyphenols have a wider range of properties, particularly tying into higher antioxidant activities, the latter have been demonstrated to have a positive effect in cholesterol metabolism and blood pressure[105]. More recent studies have started to investigate the potential neuroprotective effect of the biologically active compounds of *F.vesiculosus*, particularly pholorotannins, against Alzheimer's Disease, by inhibiting the activity of Acetylcholinesterase (AChE)[106]. Lipase activity was shown to suffer a significant inhibition using different types of extract of *F. vesiculosus* in an *in vitro* simulation of the upper digestive tract, indicating the use of this seaweed as a potential weight management tool in obesity treatments[107].

Seeing as there are many factors that affect the composition of *F. vesiculosus*, the main aim of this study is to try to shine some light into the effect some of these variables may have in important compounds and related activities in *F. vesiculosus*. Hence, the main factor under study will be the growth stage (size class) of the samples, since, as previously discussed, maturity may influence the composition of bladderwrack. Following this line of thought, gender will be the second main factor studied in this work.

1.4.1 Polyphenols in *F. vesiculosus*

Polyphenols are a wide group of phytochemicals consumed in human diet, found in many sources, from vegetables to fruits or seeds[108]. Polyphenols are secondary metabolites that are usually involved in defensive mechanisms[109,110]. As previously mentioned, in recent years, polyphenols have been an increasingly subject of interest due to their potential beneficial effects on human health, particularly related to their high antioxidant properties. Particularly in brown algae, there is a group of polyphenols that are not found in any other source and are the least studied group of tannins among polyphenols. These are named phlorotannins, which are polymeric derivatives of phloroglucinol[111]. With a molecular size up to 650 kDa, the concentration of phlorotannins generally is around 2% of the thallus dry weight, but it has been reported that in some cases it can accumulate up until 25-30%[112]. Phlorotannins are accumulated within physodes, which are cytoplasmic vesicles bound to the membrane (Figure 1.4 - b). and they can be secreted as a result of the physodes fusion with the cell membrane[113].

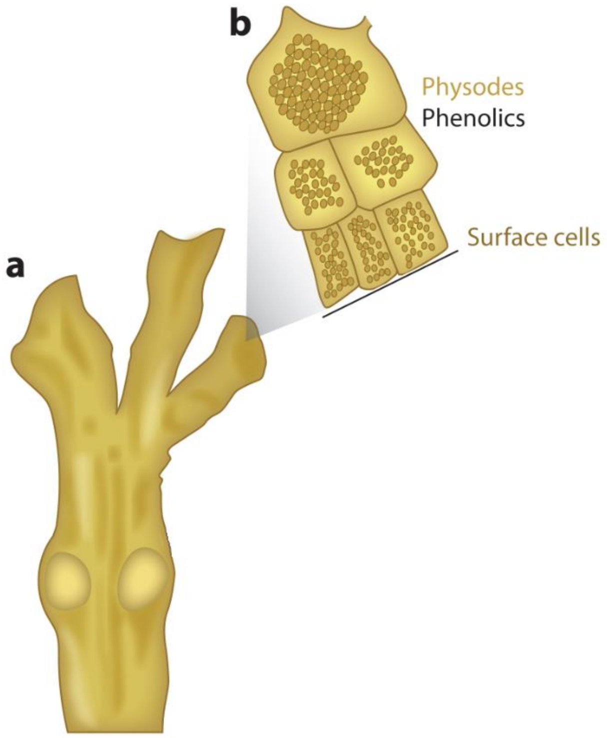

Figure 1.4. – Location of phlorotannins in *Fucus vesiculosus*[113] – (a) *F. vesiculosus* thallus; (b) phlorotannins, located within the physodes in surface cells; (c)

As other tannins, phlorotannins contain a high number of hydroxyl groups, are soluble in water, bind strongly to proteins, polysaccharides and other biopolymers, and have a polymeric structure. Their monomeric unit is 1,3,5-trihydroxybenzene (phloroglucinol) and it is thought that their biosynthetic pathway is through the acetate-malonate (polyketide) pathway. In short, with the addition of carbon dioxide, two molecules of acetyl co-enzyme A are converted into malonyl co-enzyme. Three malonyl CoA units form a polyketomethylene chain which then undergoes a Claisen-type cyclization reaction to produce a hexacyclic ring system – a triketide. Thereafter, the triketide goes through tautomerization to originate phloroglucinol, which is more stable thermodynamically. This series of reactions are conducted by an enzyme complex that combines the polyketide synthase and polyketide cyclase activities which convert acetyl CoA and malonyl CoA into the final products while not generating any intermediates[112,114] (Figure 1.5).

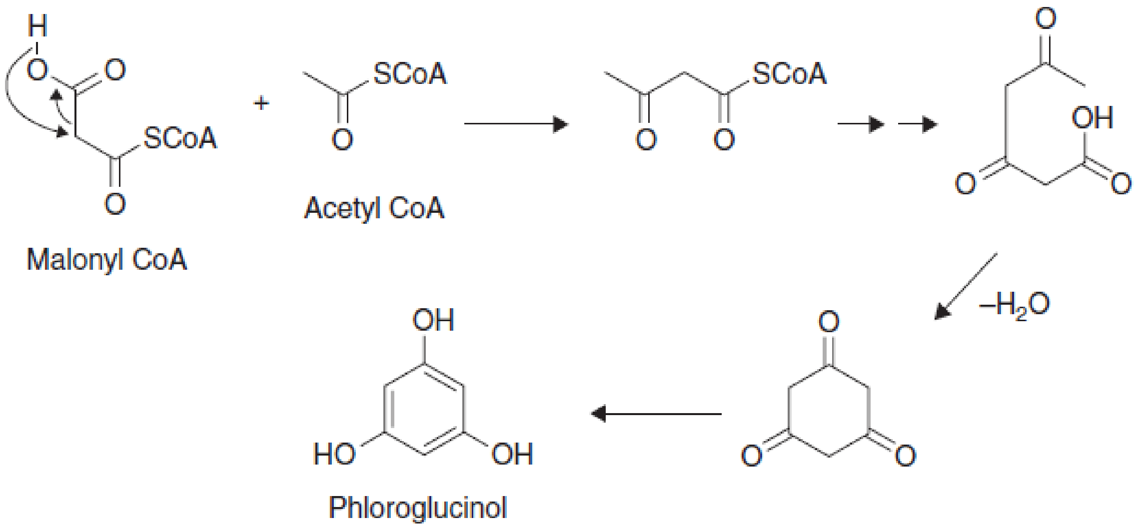

Figure 1.5. – Biosynthetic pathway of phloroglucinol[112]

The polymerization of phloroglucinol monomers occurs through C-C and/or C-O-C oxidative couplings to originate phlorotannins that can have diverse structures[111]. Depending on the linkage of the monomers, phlorotannins can be divided into four classes: (a) phloretols, with ether linkage, (b) fucols, with phenyl linkages, (c) fucophlorethols, with both ether and phenyl bonds and (d) eckols with a dibenzo[1,4]dioxin linkage. Less common types of phlorotannins include fuhalols, carmalols and compounds with benzofurodibenzo[1,4] dioxin moiety[112,115] (Figure 1.6). In each class, the linkage of monomers can take place at different places in the phloroglucinol ring, causing the formation of structural isomers, on top of conformational ones. Furthermore, certain types of phlorotannins seem to be characteristic of alga of different families - it appears that fucols and fucophlorethols are characteristic of the family Fucaecae[116] , whereas phloretols and eckols are characteristic of the families Laminariaceae and Lessoniaceae[117] , for example.

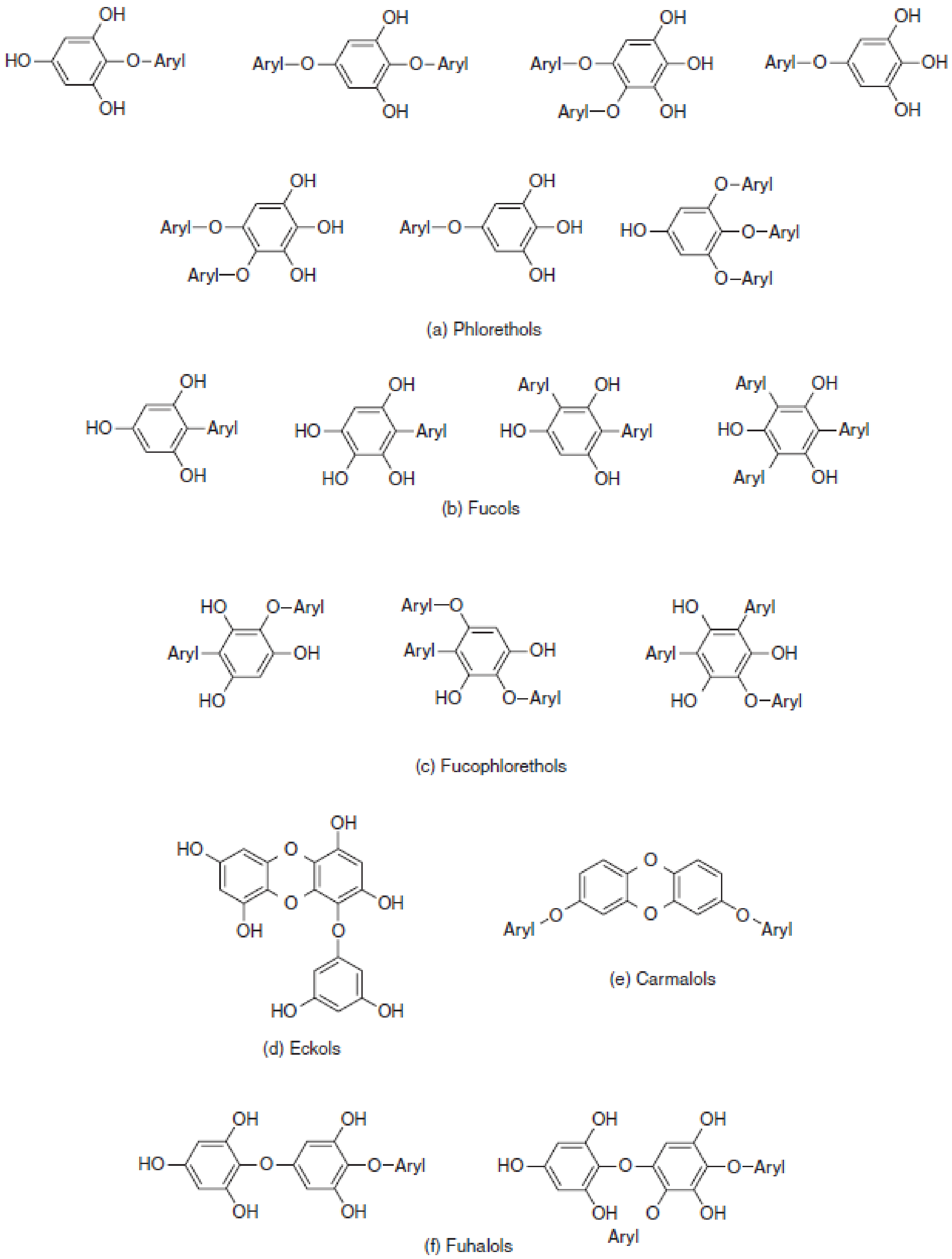

Figure 1.6. – Different classes of phlorotannins, as represented by Singh and Sidana (2013)[112].

1.4.2. Antioxidant activity in *F. vesiculosus*

The research on the use and properties of antioxidants in human health is an always increasing topic of interest since its beginning. Normally, in the process of normal aerobic cellular metabolism, free radicals, such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are generated as by-products. The most dangerous of these free radicals, in many diseases, are ROS, in particular superoxide anion (O_2^-) and hydroxyl radical (OH)[118]. As such, the mechanism of defense of antioxidants in humans plays an important role in keeping the adverse effects of these free radicals in check. As a result, a debilitated antioxidant system, associated with an overproduction of ROS often leads to oxidative stress within the organism[119].

Oxidative stress may lead to various chronic and degenerative diseases, particularly:

(i) cancer, as oxidative stress may damage the formation of hydroxyled bases of DNA, which is an important event in chemical carcinogenesis[120] and can cause genetic mutations and affect normal gene transcription and thus interfere with normal cell growth;

(ii) cardiovascular diseases (CVD), although associated with a multitude of other risk factors in its development (e.g. hypercholesterolemia, hypertension, smoking), research suggests that CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure may be associated with oxidative stress[121,122];

(iii) neurological diseases, such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss or depression[123,124];

(iv) certain pulmonary diseases, such as asthma, chronic obstructive pulmonary disease (COPD) – oxidants may enhance inflammation by activating different kinases and redox transcription factors in these diseases that are characterized by systemic and local chronic inflammation[125,126];

(v) rheumatoid arthritis, as the pathogenesis of this disease is caused by generation of ROS and RNS at the sites of inflammation[127]; (vi) renal diseases, such as glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria and uremia[122];

(vi) ocular diseases, such as cataracts, by altering cell types in the eye photochemically or nonphotochemically – formation of cataracts may be formed through the aggregation of crystalline protein in the lens under the action of free radicals[128] and finally

(vii) oxidative stress may impact various mechanisms in the development of fetal growth restriction and pre-eclampsia in prenatal medicine as some studies suggest that ROS and RNS may have a role in the etiology of pre-eclamptic pregnancies[128].

A brief resume of the diseases discussed in which oxidative stress plays a role is compiled in Figure 1.7.

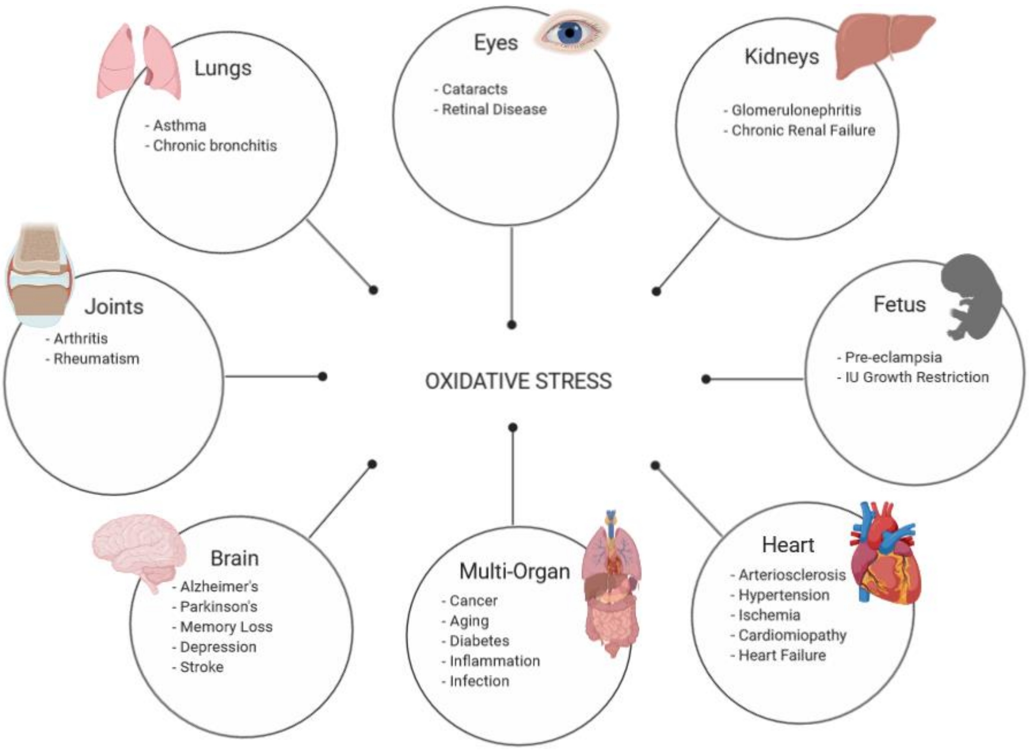

Figure 1.7. – Chronic and degenerative diseases induced by oxidative stress. Adapted from Pham-Huy et al.[121]

As a result of the problems that stem from oxidative stress and in an attempt to prevent them, the supplementation with antioxidants to complement organism's antioxidant defense system, through its various sources, either synthetic or natural is important, especially in groups that are more susceptible to an imbalance. Therefore, extensive research has been made in the effects on external antioxidant supplementation to evaluate their effectiveness. Currently, one of the biggest challenges in this area is to establish biomarkers for oxidative/nitrosative damage in both animals and humans based on specificity, reproducibility and causal relation with disease and how they respond to supplementation with antioxidants both short and long term[118].

Antioxidant originating from natural sources tend to be the preferable choice in supplementation for prevention of oxidative stress induced diseases, not only because they may work as natural antioxidants in their own systems *in vivo* but also because they also bring additional health benefits, such as acting as inducers of mechanisms regarding antioxidant defense, longevity and DNA repair[129–131]. Therefore, natural sources of antioxidants have been a topic of research with a growing interest in recent years, in an effort to better grasp their influence. Summarily, natural antioxidants such as vitamin (C and E), carotenoids (β-carotene, lycopene and astaxanthin), Polyphenols (tea polyphenols, red wine polyphenols and chocolate polyphenols) and flavonoids (flavonoids, isoflavones, xanthones and anthocyanins) have all well-known effects in chronic diseases associated with oxidative stress[132]. Particularly, polyphenols, derived of their chemical structures with multiple hydroxyl groups, make them good electron and hydrogen atom donors and thus excelling at neutralizing ROS and other free radicals[133].

As a brown alga, *F. vesiculosus*, contains high levels of polyphenols, especially phlorotannins, making it a suitable candidate for antioxidant supplementation. Although some studies have been made in an effort to study this potential, both in its own and compared to other brown algae[17,134], a comprehensive research within the bladderwrack's own potential, particularly in accessing the best conditions to achieve the highest antioxidant properties is yet to be made.

1.4.3. *F. vesiculosus* and symptom treatment of Alzheimer's Disease

One of *F. vesiculosus* potential applications, that in recent years has been scrutinized, is in Alzheimer's Disease treatments. Alzheimer's Disease (AD) is a type of brain disease that damages and destroys neurons involved in cognitive functions, such as thinking, planning, learning and memory and it usually originates years before any kind of symptoms may be acknowledged by a pacient[135]. Being a neurodegenerative disease, it progresses over time, from interfering with day-to-day activities (e.g. planning events, forgetting about tasks) to basic bodily functions in the most advanced stages of the disease[135]. To this day, dementia caused by AD has no known treatment capable to stop or slow the damage of neurons associated with Alzheimer's symptoms. Current available drugs only alleviate some symptoms of the disease[136]. Future treatment options to slow this progress are believed to be most effective when administrated during the earlier stages of the disease, either at a pre-clinical stages or mild cognitive impairment (MCI) Alzheimer's with aid of biomarkers to identify patients in early stages and monitor the progression of the disease and the efficacy of the administrated treatments[136].

One of the most common brain changes that occurs in AD is the accumulation of beta-amyloid (amyloid hypothesis) outside the neurons that can contribute to cell death by interfering in neuron-to-neuron interaction at synapses. Other brain changes may be related to the accumulation of tau tangles, an abnormal form of tau protein, inside the neurons that can block the transport of essential nutrients and molecules inside the neurons (tau hypothesis)[135,137]. Another hypothesis, the cholinergic hypothesis suggests that a loss of cholinergic functions in the Central Nervous System contributes significantly to the degradation of cognitive functions associated with AD[138]. In AD, there usually is a deficiency in acetylcholine (ACh), a molecule that mediates neurotransmission in the brain[139]. As a result, one of the most recent approaches to the symptomatic treatment of AD involves the inhibition of the acetylcholinesterase enzyme (AChE), that catalyzes the breakdown of AChE[139,140](Figure 1.8).

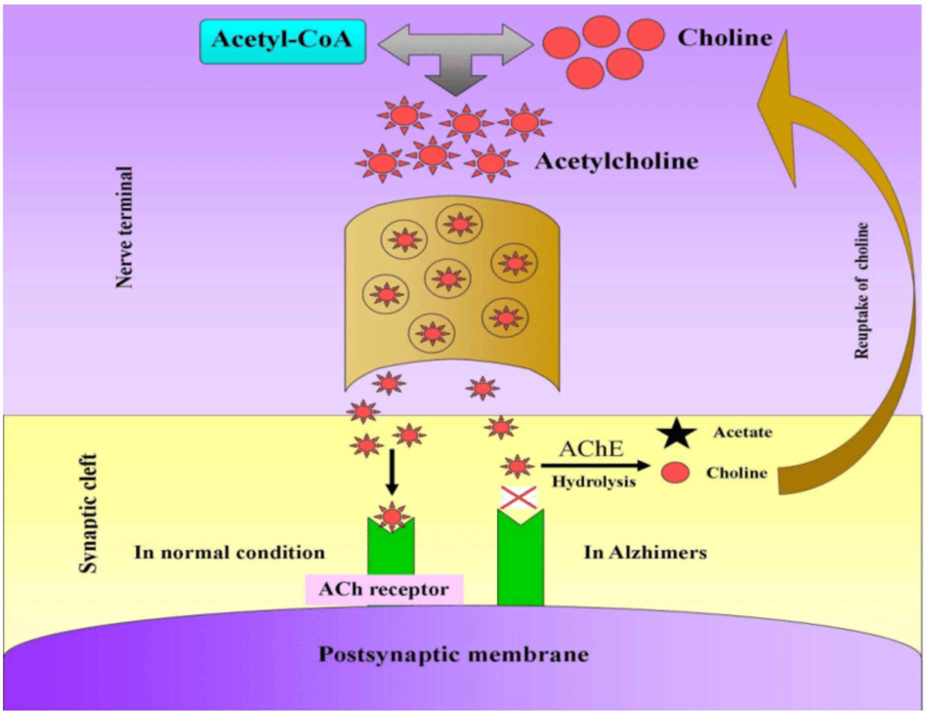

Figure 1.8. – AChE mecanism of action in normal conditions and in Alzheimer's Disease[140].

Seaweed-derived biologically active compounds have been found to exhibit inhibitory effects on the activity of AChE and butyrylcholinesterase (BChE), particularly in extracts rich in phlorotannins and flavonoids from *Ecklonia maxima*, *Gelidium pristoides*, *Gracilaria gracilis* and *Ulva lactuca*[141,106]. Sulfated polysaccharides from these species, as well from *Ulva rigida* also showed inhibition towards AChE and BChE[142]. The inhibitory effects of eight south African seaweeds, *Codium capitatum, Codium duthieae, Halimeda cuneata, Ulva faciata, Amphiroa beauvoisii, Gelidium foliaceum, Laurencia complanata* and *Rhodomelopsis africana* were also analyzed, and albeit at lower percentage levels, all seem to show inhibitory activity of different degrees. In the same study, the phenolic, flavonoid and condensed tannin levels were determined and the variations between the inhibitory activity that each species shows may be attributed to differences in their constitution, in particular their flavonoid and alkaloid compositions[143]. Furthermore, different types of compounds extracted from seaweed suggest an inhibitory activity on AChE and BChE, specifically, glycoproteins isolated from *Undaria pinnatifida*, in which higher inhibitory effects were obtained on AChE, when compared to BChE[144]; bioactive compound alpha bisabol present in *Padina gymnospora*[145] and fucosterol isolated from *Sargassum horridum*[146], as well as photofucofuroeckol isolated from *Ecklonia cava*, where the latter showed a higher inhibitory effect against BChE than AChE[147].

In *F. vesiculosus*, whilst very few studies have been done regarding its properties in AChE activity inhibition, and thus there is the need to shine some light in this subject, fucoidans from these species show an inhibitory effect on Aβ aggregation, one of the main brain changes discussed in this chapter, and reduction in the cytotoxicity of $A\beta_{1\text{-}42}$ and hydrogen peroxide in neuronal PC-12 cells, as well as inhibition of apoptosis induced by $A\beta_{1\text{-}42}$[148].

1.4.4. *F. vesiculosus* mineral composition and its applications

The mineral composition of a product like a seaweed-based supplement is very important and it has many different applications, not only as a product *per se*, depending on the essential minerals and trace elements that constitute them and the nutritional value associated but because they can also serve a biological purpose, such as acting as bioindicators for the presence of heavy metals in certain habitats, such as estuaries. Furthermore, different genera of seaweeds vary in elemental composition and even within the same species, seasonal differences, different geographic locations, light intensities or different seaweed type (wild vs cultivated) can influence their final mineral constitution[149]. As such, analyzing the different minerals present in a certain seaweed and its variations is not only important to fully take advantage of all their properties but it is also useful to identify the presence of potential harmful ones, associated with the growth conditions cited above. A particular characteristic of algae is their wide variety and high level of mineral (8-40)% that is related to their capacity to retain inorganic marine substances[150]. In addition, their mineral content, traduced by their higher mean ash contents is generally higher than terrestrial plants and other animal products[151].

F. vesiculosus, as other species within its genera, contain several important elements, in particular, Na, K, I, Ca, Fe, Mg, Cu, Zn and Mn (Table 1.1 presents mineral compositions from *F. vesiculosus* harvested from different regions). Compared to terrestrial sources and even within *Fucus* spp., *F. vesiculosus* is considered an excellent source for iodine, an element that is important in the production of thyroid hormones, which can be useful to individuals with an iodine deficiency[101].

Table 1.1. – Mineral composition of different samples of *F. vesiculosus* harvested from various regions across Europe. n.d.: not determined.

Mineral Composition of various samples of *F. vesiculosus* in different regions					
	Norway[152] (mg kg−1 DW)	**Coruña, Spain**[101] (mg kg−1 DW)	**Jūrmala, Latvia**[153] (mg kg−1 TS)	**Baltic Sea, Estonia**[154] (mg kg−1 DW)	**Pontevedra, Spain**[103] (mg kg−1 DW)
Ca	12000	11602.7 ± 231	21500	1200-5400	9380 ± 70
Na	n.d	21875 ± 369	6300	480	54690 ± 600
Mg	7400	7323 ± 535	9300	5100	9940 ± 130
P	840	1935 ± 11	1400	n.d	n.d
K	n.d	37450 ± 360	11000	2000-7000	43220 ± 460
Cu	1.8	n.d	12.7	20	<5
Fe	92	189.9 ± 3.2	490	280-320	42 ± 1.7
I	130	n.d	n.d	130-160	n.d
Mn	34	82.8 ± 10	1680	415	55 ± 1.1
Se	0.03	n.d	0.11	n.d	n.d
Zn	26	n.d	89	280	37.1 ± 3.7
As	41	n.d	13.5	330	n.d
Cd	1.2	n.d	1.7	n.d	n.d
Hg	0.011	n.d	n.d	0.0063-0.0070	n.d

Additionally, even though the concentration of Na varies largely between the data collected over the different harvest sites, the Na/K ratios are generally on the lower end of the spectrum. This is particularly interesting from a nutritional point of view, as high Na/K ratio diets (common in modern Western diets) and the incidence of hypertension are highly correlated. Therefore, products with low Na/K ratios, as *F. vesiculosus* may constitute a good alternative to regulate levels of Na/K. Regarding other major minerals, in general, concentrations of Ca and Mg are higher than those found in other common products, such as milk, for Ca or peanuts for Mg. On another note, for the elemental analysis in *F. vesiculosus* harvested from Estonia (Neutron activation analysis - NAA) has low sensitivity for K and Ca, as is reported by the authors[154] although these elements, as described through other studies, are normally major components of bladderwrack mineral composition.

Although there is a consensus that *F. vesiculosus* has many positive properties associated with its consumption, algae are also known to for their ability to bioaccumulate heavy metals, which can have a harmful impact in human health. As a result, studying the accumulation of heavy metals in *F. vesiculosus* is important in two main aspects: (i) in a biological standpoint, it helps in verifying the adaptability of the species in different habitats, that may be exposed to different types and concentrations of heavy metals and thus its role in serving as a bioindicator of these habitats; (ii) in a nutritional standpoint, these kind of studies are fundamental to determine if *F. vesiculosus* harvested from a certain habitat can be converted in a product available for human intake, namely to assure the concentration of heavy metals in their constitution is safe for consumption – limits for the safe intake of this type of contaminants have been set by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and are expressed by the provisional tolerable weekly intake (PTWI) of a certain heavy metal (usually in μg kg^{-1} body weight per week)[152].

As previously discussed, the concentration of heavy metals in seaweed can vary according to numerous factors, being the most common As, Cd, Cu, Hg, Pb and Zn. The *Fucus* spp. is especially subject to accumulate heavy metals as they can tolerate high levels of these contaminants. In addition, their inability to regulate the concentrations of heavy metals in their tissues makes them adept in their bioaccumulation in contaminated habitats[155]. Furthermore, excluding the variation offered by different habitats, the physical state in which a heavy metal is present in the composition of *F. vesiculosus* is also important to note. For example, As has been found to be present in a high concentration in samples of *F. vesiculosus* harvested in the coast of Norway[152], however, many studies have found that algae in *Fucus* spp. are capable of metabolizing the inorganic form of arsenic, in its most toxic state, to its organic form[156,157]. As such, even though this heavy metal may be present in high levels it does not necessarily mean that its poisonous - thus, in addition the study of these pollutants composition, it is also beneficial to study the speciation of their chemical forms, if need be.

Regarding its potential as a bioremediator, *F. vesiculosus* may constitute a good natural renewable alternative accumulator that may be introduced in water treatment technologies. Cadmium, one of the most toxic heavy metals found in polluted estuaries, has been shown to be efficiently removed from wastewater by *F. vesiculosus* from the Bothnian Sea, an effect that may be attributed to the alga's surface charge characteristics, such as faster deprotonation capacity and higher number of acidity groups[158]. This study also points out the effect of salinity in metal adsorption and the photosynthetic response of the alga, claiming that treatment cycles should focus on a lower salinity system, as the removal capacity of heavy metals appears to be inversely proportional to the ionic strength.

In a similar light, to study the effects of environmental pollution on estuaries and the coastline, biomarkers that provide a clear indicator of this kind of contamination are extremely useful. In the case of heavy metals' assessment, *F. vesiculosus* from the Eastern Scheldet Estuary may serve as a good biomarker, as long-term exposure to high concentration of heavy metals has an effect in the pigmentation of individuals sampled from this estuary[159]. This happens because certain heavy metals, such as the ones utilized in this study (Cu^{2+} and Cd^{2+}) substitute the central atom of chlorophyll, Mg^2, which results in a decrease in fluorescence quantum yield and a shift in the fluorescence spectrum[160]. This pigment changes due to heavy metal presence, being easily detectable, make *F. vesiculosus* a good candidate as a biomarker.

Particularly to the Portuguese coastline, the Tagus estuary, being one of the biggest in Europe with 320 km^2, has a history of being subject to continuous pollution, directly receiving effluents from agricultural, industrial and urban sources[161]. In addition, effluents stemming from the approximately 2.5 million Greater Lisbon inhabitants negatively impact the commercial and fishing activities in the estuary. Furthermore, industrial areas located in the north and south margins, as well as domestic effluents from the metropolitan area have also contributed to the estuary's contamination. These events have made the Tagus Estuary a sink for heavy metals[162], and therefore many efforts have been made in order to study the effects of heavy metal accumulation in this area. However, little is yet known about the effect of these contaminants in *F. vesiculosus* and thus the present works aims to shed light regarding this topic.

2. Objectives

The aim of this work is to elaborate a characterization of *F. vesiculosus* that inhabits the Tagus Estuary. In particular, the main purpose of this study is to investigate the questions of whether *F. vesiculosus* fronds of different growth stages (size classes) and gender show differences in the following aspects related to their biochemistry:

(i) Total Phenolic Content (TPC), measured spectrophotometrically using an adapted method utilizing Folin–Ciocalteu reagent and phloroglucinol as the standard;
(ii) Antioxidant activity, as the capacity to reduce the DPPH radical, and how its correlated with the TPC;
(iii) To study the composition of *F. vesiculosus* and how it differentiates during the alga's development and between genders using High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD);
(iv) Inhibition towards acetylcholinesterase (AChE), to study the potential role of *F. vesiculosus* in the treatment of symptoms of Alzheimer's Disease;
(v) Mineral Composition, to identify the benefic nutrients present in *F. vesiculosus* individuals from Tagus estuary, as well as heavy metals that may be present in harmful concentrations and to access its potential as a biomarker or bioremediator.

With these five main topics of study, the present work seeks to, in addition to the biochemical analysis aforementioned of *F. vesiculosus* from the Tagus estuary, to try to find the optimal conditions of stage of growth for harvesting for the populations growing in this area.

3. Material and Methods

3.1. Sampling

Sampling of *F. vesiculosus* was done in the Tagus Estuary (38°46'56.2"N 9°05'27.7"W), in October 10th 2018. The algae samples were collected and stored at low temperature (4º C). Four classes of *F. vesiculosus* were then created, according to the growth stage each sample belonged to, corresponding to the length of the thallus (<20 cm; 20-25 cm; 25-30 cm; >30 cm size classes). Each sample was then measured and grouped as described, matching with the size class they fitted in.

Each size class sample was then subdivided according to their sex, by observation through a magnifying hand lens or a microscope: (i) manual transverse sections of the receptacles were cut and observed to identify the presence of the large, dark oogonia, indicative of mature female conceptacles/fronds, which were separated, then, (ii) to differentiate between non-mature females and males, the same type of transverse sections were done and observed in the microscope, at 40 x magnification, to positively identify the presence of male reproductive organs, the antheridiophores. All samples, now grouped by size class and sex, were then stored at 4º C until further processing (Figure 3.1).

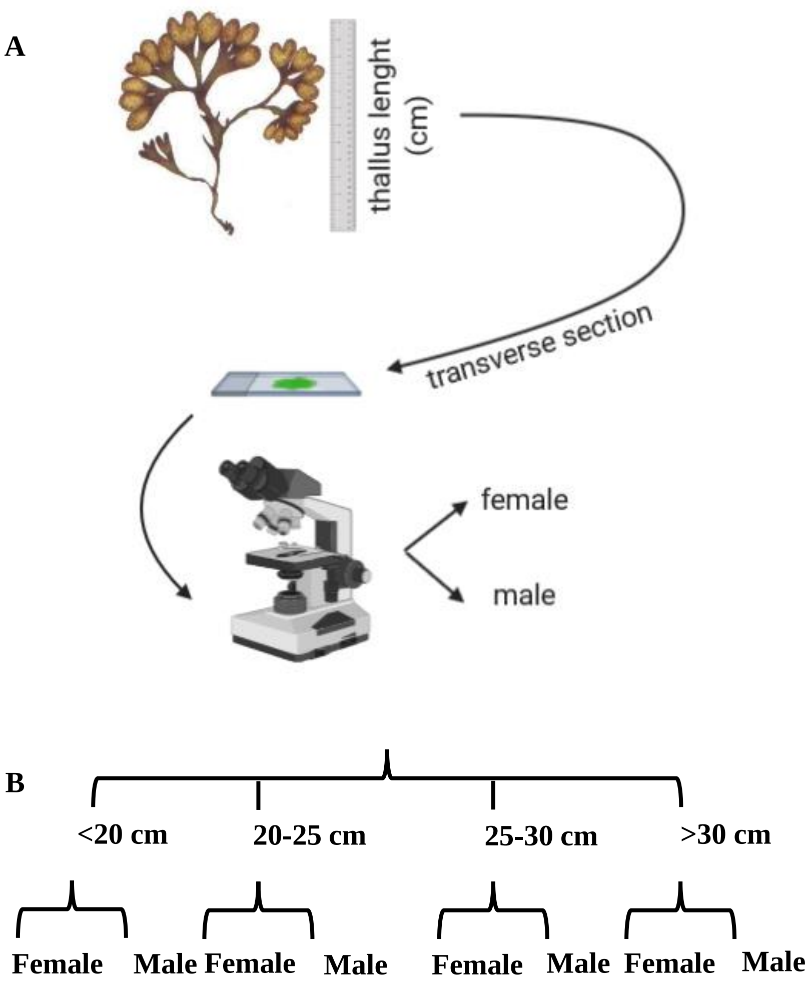

Figure 3.1. - Brief schematics representing the sampling process. (A) First, thallus length was measured, following a microscopic analysis to differentiate between genders. (B) The division of the samples is represented in the second schematic – four groups representing four different growth stages (size classes) subdivided by their gender, for a total of eight subsamples destined for biochemical analysis.

3.2 Extractions

After the subsampling process, the material was lyophilized: The samples were frozen for a 24 h period, and then lyophilized (Alpha I-5 Christ) at 10^{-1} mbar and -42º C, for 3 days. Samples were then stored at -20 ºC, until analysis.

Extractions were made in order to mimic the way one would normally consume *F. vesiculosus* – by doing so, it is possible to analyze the components and properties of each type of extract, as well as being able to determine their differences. Two types of extraction were performed, albeit both being aqueous extractions. For each sample, both types of extraction were made, obtaining 16 samples in total.

3.2.1 Aqueous Extraction at 100 ºC

Each sample of lyophilized F. *vesiculosus* was pulverized and 5 g of this seaweed were weighted (Sartorius® BP 110 S scale) and mixed with 100 mL of distilled water in a volumetric flask and then placed on a heated plate at 100 ºC, for 30 min (ARE Heating Magnetic Stirrer VELP Scientifica ®).

3.2.2 Aqueous Extraction at room temperature

A similar type of extraction was also carried at the same time – 5 g of algal powder was measured and mixed with 100 mL of distilled water and placed on a platform shaker at 200 rpm, during 24 h and at approximately 25 ºC.

After extractions, both type of mixtures were centrifuged at 3500 rpm for 10 min at 4 ºC (JA-20 rotor using a Beckman® J2-21M/E Centrifuge). The supernatants of these extracts were then lyophilized, registered and stored at -20 ºC [163],[164].

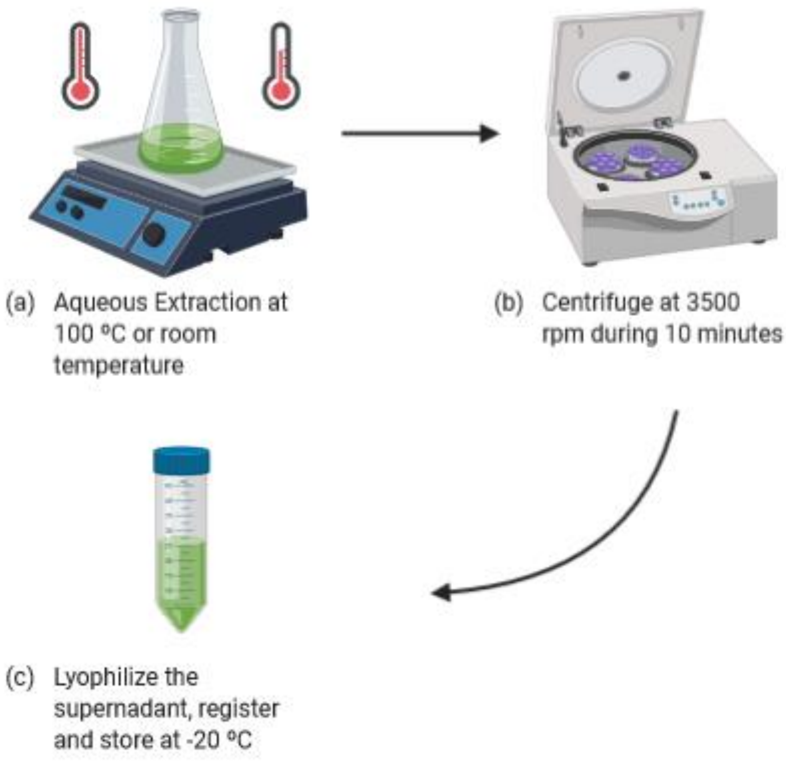

Figure 3.2. - Schematic representation of both types of extractions of *F. vesiculosus* described

3.3 Total Phenolic Content Analysis

Total phenolic content (TPC) was measured spectrophotometrically using Folin–Ciocalteu reagent and phloroglucinol as the standard (calibration curve 10 to 1000 µg/mL), in a slightly modified protocol of Henriques et al [163]. 30 µL of seaweed extract (at a 10 mg of extract/mL) was added to 1270 µL of distilled water. 30 µL of Folin–Ciocalteu were then mixed with this solution and, after 5 min, 90 µL of a 2% solution of sodium carbonate were added. The samples were then heated at 60 ºC for 30 min in a water bath Butchi ® B-490. The absorbances (A) were then measured, in triplicates, at 760 nm in a Shimadzu ® UV-160A spectrophotometer. The concentration of total phenolic content was calculated as micrograms of phloroglucinol equivalents, as the mean of the three replicates, per milligram of dry seaweed extract (µg/mg DW). Hence, the following equation was used to determine TPC for each replicate:

3.1 - $$TPC = \frac{Asample - yintercept}{Slope} \times Vcuvette \times Veppendorf \div Vsample \div Csample$$

Where TPC corresponds to the Total Phenolic Content (µg of phloroglucinol equivalent in a mg dry seaweed extract); A_{sample} to the absorbance of the sample; $y_{intercept}$ to the intercept of the calibration's curve equation obtained; Slope to the value of the slope of the same equation; $V_{cuvette}$ to the volume of the solution present in the cuvette, utilized during the absorbance readings; $V_{eppendorf}$ to the volume of the solution mixed in an Eppendorf; V_{sample} to the volume of sample that was mixed in the solution (30 µL), and C_{sample} to the concentration of the extract's solution, which was utilized in this essay (10 mg/mL). This was repeated in all triplicates, and the mean between these was determined as being the concentration of total phenolic compounds present in the particular sample. This essay was carried out for the samples obtained in the two types of extractions done, throughout all four growth stages and two genders.

3.4. Antioxidant activity Determination

In order to determine the antioxidant properties of *F. vesiculosus* and potential differences through growth stages and gender, an essay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging radical, was carried out to evaluate the capacity of *F. vesiculosus* to reduce the DPPH radical. An adaptation of the DPPH method described by Falé et al. (2013) was utilized [165]. 10 µL of sample at a concentration of 10 mg/mL were mixed with 1 mL of DPPH (0,02 mg/mL in MeOH). Blank solutions were composed by 1 mL of DPPH mixed with 10 µL of distilled water. The solutions were then exposed at room temperature for 30 min. Absorbance of the different samples was then read on the spectrophotometer at 517 nm nm in a Shimadzu ® UV-160A spectrophotometer. The essay was repeated in triplicates and the data presented is shown as the means and standard deviations of the measurements obtained. The percentage of antioxidant activity was obtained through the following formula A_{sample}

3.2 - $$AA (\%) = [\frac{A\ sample}{A\ \text{DPPH Solution}} \times (-1) + 1] \times 100$$

Where AA (%) corresponds to the percentage of antioxidant activity, A_{sample}, to the absorbance of each sample and $A_{\text{DPPH Solution}}$ to the absorbance of the DPPH solution.

3.5 High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD)

Both type of extracts prepared were subjected to a chromatographic analysis conducting a High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD). 1 mg/mL of each extract was analyzed in a VWR-Hitachi Elite LaChrom® equipped with a LiChroCART®RP-18, 5µm, 250×4 mm, 100 Å column from Merck, autosampler L-2200, column oven L-2300 and diode array detector (DAD) L-2455. In this step, the mobile phase consisted 0.05% (v/v) of trifluoracetic acid

(Merck) in water and acetonitrile (Carlo Erba). Detection was carried out between 200 and 600 nm using diode array detector (DAD) and data acquisition was carried out using EZChrom Elite ®, Hitachi Japan software.

3.6 Acetylcholinesterase inhibition

An essay to determine the *F. vesiculosus* extracts inhibition towards acetylcholinesterase was carried out following a similar protocol described by Falé et al. (2013) [165]. Firstly, 325 µL of Tris buffer (50 mM) at pH 8 are mixed with 100 µL of sample (10 mg/mL) and 25 µL of acetylcholinesterase solution in a 1.5 mL cuvette. This solution then rests for 15 min at room temperature. Then, the cuvette is placed in the spectrophotometer, in which 75 µL of AChl solution are added, as well as 475 of 3 mM Ellmen's reagent (DTNB). The absorbance is then read during the first 5 min of the reaction at 405 nm. Each time this essay was carried out, a control reaction was also performed, replacing the 100 µL of sample for the same volume of distilled water.

3.7 Heavy Metals Identification Essay

Quantification of total elemental concentration in the sampled seaweed was done using Total Reflection X-Ray Fluorescence Spectrometry (TRXF). *F. vesiculosus* lyophilized samples (≈ 180 mg) were mineralized with 2 mL of $HNO_3/HClO_4$ (7:1 v/v) at 110 º C for 3 h in a Teflon reactor [166]. Ultra-pure acid Gallium solution (IS) is then added to the mineralization samples. Following this addition, 5 mL of each obtained samples are placed in an individual siliconized quartz disc and dried for 15 min on a hot plate set at 80º C for a total of 6 samples, with three replicates each. After this step, the final samples for this analysis contained thin, circular seaweed films on the quartz glass sample carriers. Samples were then measured in triplicate in the TXRF and the relative abundance of each element was calculated. In this process, cadmium was included for spectra correction of certain detector artifacts, serving as a 1 gain correction mono-element standard sample for each replicate batch. Although *F. vesiculosus* has been studied for its Cadmium adsorption [158,167], this element was not the main focus of this analysis. The software SPECTRA 6.3 (Bruker AXS Microanalysis GmbH) was utilized for data evaluation and TXRF spectra analysis [168]

Concerning the decontamination procedures prior to the preparation of TXRF quartz glass sample carriers, the following procedures were taken into account in the cleaning of these carriers: (i) each sample carrier was wiped with a fluff-free tissue paper soaked in pure acetone, (ii) the sample carriers were mounted onto a washing cassette which was then transferred into a beaker containing alkaline cleaning solution (RBS 50 (Chemical Products S.A., Belgium) diluted 5:50, RBS:ultra-pure water), (iii) using deionized double distilled water to wash the sample carriers and consequently placing them again in the washing cassettes into a beaker filled with 10% nitric acid (Sigma-Aldrich, Germany), (iv) carefully rinsing the sample carriers with ultra-pure water, (v) in an oven set at 80º C, drying the sample carriers for 30 min and proceeding to cautiously wipe them with tissue paper soaked in acetone, and lastly (vi) adding 10 µL silicon solution (Serva Electrophoresis, Germany) at the center of the quartz discs on a hot plate, as to leave a hydrophobic surface residue of silicon that assists to center the sample in the carriers for the TXRF analysis. All washing steps were performed in a microwave for 5 min at 800 W.

Regarding TXRF specifications, the particular instrument utilized (S2 PICOFOX spectrometer) has the ability of multi-elemental analysis which allows for the detection of a great number of elements present in the *F. vesiculosus* samples. A rundown of its specifications are presented in table 3.1. [169]

Table 3.1. – Technical specifications of the S2 PICOFOX TXRF spectrometer.

X-ray tube	50 kV, 1mA, Mo target
Element range	Na to U
Optics	Multilayer monochromator, 17.5 keV
Detector, area, resolution	Silicon drift, 10 mm^2, <160 eV
Carrier	Quartz or other material, 30 mm diameter
Sample station	Cassette for 25 disks
Control	PC, data transfer via serial interface
Size, weight	590 x 450 x 300 mm, 37 kg
Power consumption	Max 150 W
Voltage, frequency	100-230 V ± 10%; 50-60 Hz
Manufacturer	Bruker AXS Microanalysis GmbH

3.8. Statistical Analyses

Results were expressed in terms of mean ± standard error of the mean (SEM). Statistical comparisons were performed using a Two-Way ANOVA with Bonferroni Correction Test on GraphPad 8.3.0.

4. Results and Discussion

F. vesiculosus is a seaweed rich in polyphenols [170], particularly phlorotannin[171] which contribute to its inherent antioxidant properties[171,172], antitumoral activity[173] and antibacterial effect[174]. However, few studies review more in the depth the antioxidant potential of these compounds, especially when comparing between growth stages. As such, one of the main objectives of this work was to study the potential differences along this alga's life cycle, in both genders. Furthermore (as we also aim to study its potential health benefits), it is also relevant to study the way we consume this seaweed, as it may be relevant to which compounds are exactly present in the extracts we eat.

Besides these main objectives, we also set to study the alga's components throughout its growth, as well its potential use as an inhibitor towards acetylcholinesterase. Lastly, as our samples were collected in an area historically susceptible towards river pollution [175], we set to evaluate the presence of heavy metals in *F. vesiculosus* that could be potentially harmful to human consumption, as well as the presence of important nutrients in this seaweed.

In short, the work presented in this thesis aimed to identify the best possible state in which *F. vesiculosus* should be harvested.

4.1. Total Phenol Content

Past works with brown algae show that density in physodes and, consequently concentration in phlorotannins, varies within-thallus, as apical, younger portions of the thallus tend to exhibit lower values when compared to the basal portions[176]. Furthermore, and although previous studies analyze the total phenol content in *F. vesiculosus* [134,171], the characterization we aimed to carry out intended to differentiate the TPC between female and male alga of different thalli length (<20 cm, 20-25 cm, 25-30 cm and >30 cm) and two different type of extraction (100 ºC and room temperature), totaling 16 samples to analyze. As such, in this step of the work we calculate the TPC for each of the aforementioned samples, as explained in chapter 3.3. The resulting values were then organized for ease of comparison as presented in Figure 4.1 and 4.2. The values measured spectrophotometrically, as well as the detailed values for TPC.

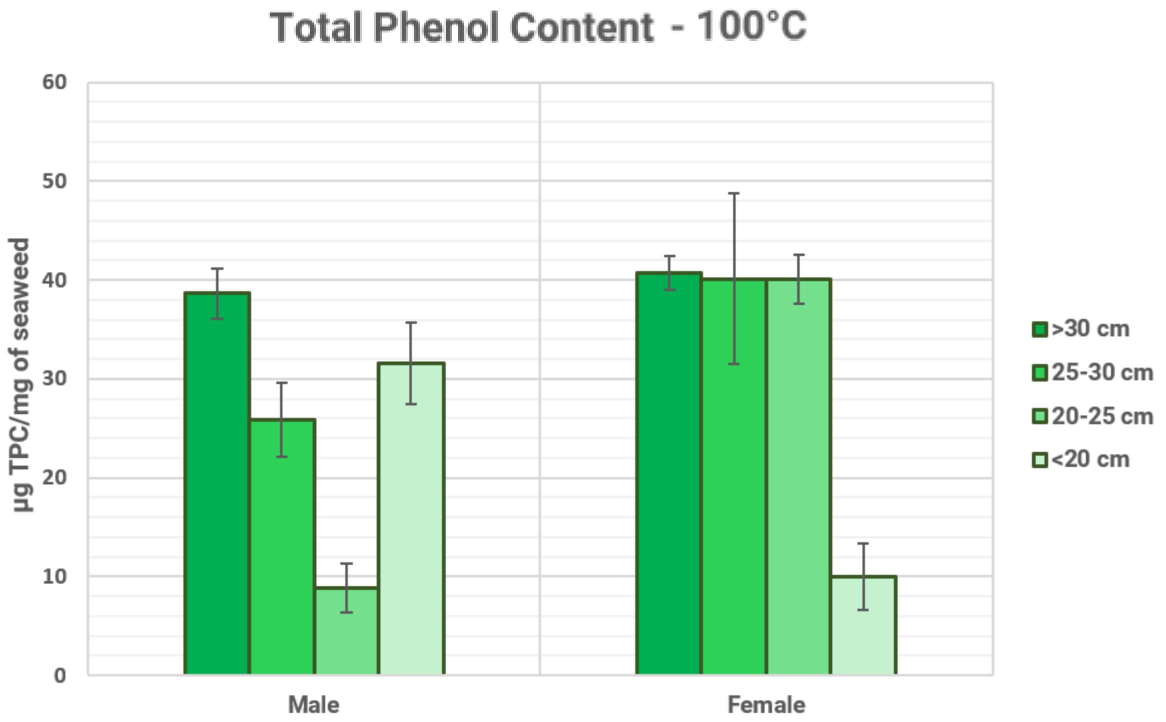

Figure 4.1. - Total Phenol Content (µg) present in a milligram of a *F. vesiculosus* extract, obtained performing a decoction at 100 ºC. Data shown is relative to the amount of TPC present in males and females' samples, across the four growth stages represented with respective standard deviation.

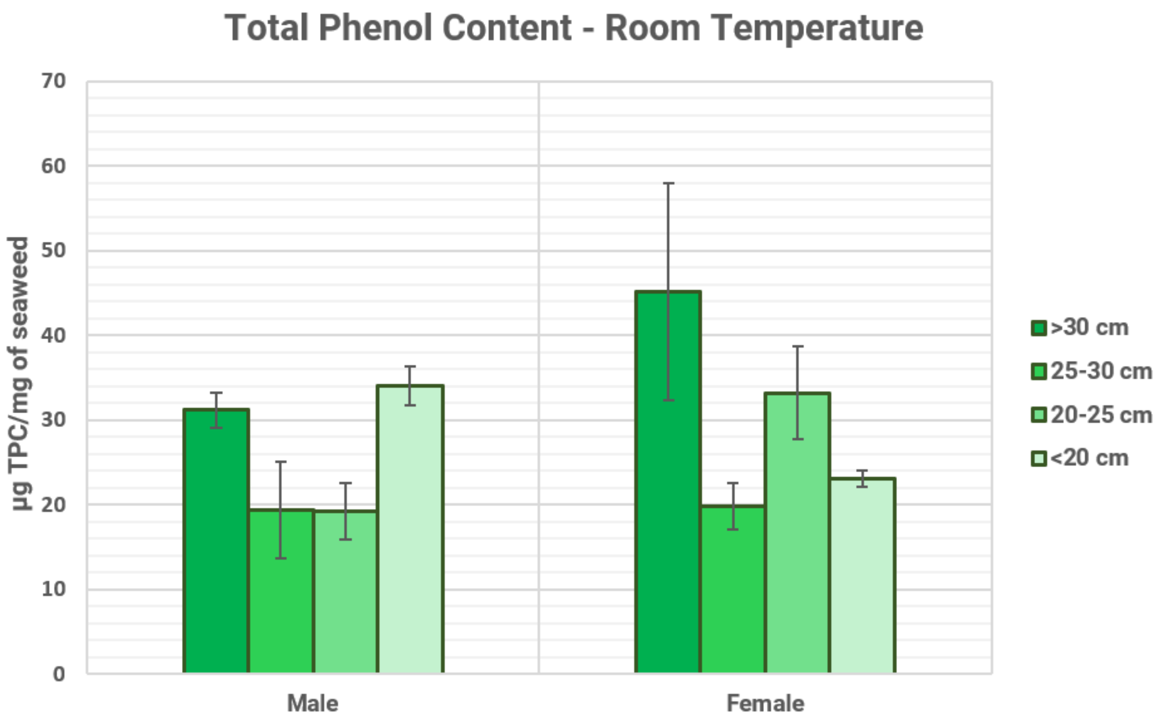

Figure 4.2. - Total Phenol Content (µg) present in a milligram of a *F. vesiculosus* extract, obtained by aqueous extraction at 25 ºC, during 24 h. Data shown is relative to the amount of TPC present in males and females' samples, across the four growth stages represented with respective standard deviation.

One pattern we immediately noticed is that the TPC achieves similar higher values in adult stages, indicating there is a certain tendency for a higher TPC to be stored at such stages. As TPC, especially phlorotannin content was shown to be higher in vegetative branches of this seaweed [177] it is reasonable that a higher length of the vegetative thalli should in turn be indicative of a higher TPC.

However, while analyzing these results, in both types of extracts, the younger male, specifically at thalli length inferior to 20 cm, showed a higher TPC. Statistical analysis further demonstrates this result, especially in the samples extracted at 100º C. Bonferroni's Correction shows a significant difference between the two youngest growth stages of males (<20 vs 20-25 cm; *p-value* = 0.0001), while also demonstrating that the youngest males have a significant higher TPC, when compared with the female counterpart (male <20 vs female <20; *p-value* = 0.0003). This might be indicative of a potential role played by phlorotannins in the early stages of *F. vesiculosus* development and sexual maturation. The tendency shown by the TPC in both genders might also indicate that the role of phlorotannins is different regarding the sexual maturation of the gametes within the conceptacle, as male gametes mature earlier and remain fertile during longer periods than female gametes.

Regarding the samples extracted at 25º C, while the differences described between the youngest male stages are not significant (as opposite to the latter set of results – extracts at 100º C) they do not exclude the hypothesis proposed as results still show a tendency to have a higher TPC. However, both intermediate male growth stages have significantly lower TPC than the adult females (male 20-25 vs female >30 cm and male 25-30 vs female >30 cm; *p-values* = 0,0011), which shows that the youngest males still have a higher TPC and, therefore, the premise suggested may still be true.

It is also noteworthy to point that the temperature of the extraction may have an influence over the TPC obtained. Although time of extraction should not cause a decrease in TPC, it was previously observed that at lower temperatures, particularly at 25 ºC, phlorotannin content is significantly lower in *F. vesiculosus* samples, when compared to samples extracted at higher temperatures [178].

4.2. Antioxidant Activity Determination

In the second step of our characterization, we wanted to further study the antioxidant properties of the different stages of *F. vesiculosus*, and how it compared to the results of the phenolic contents previously obtained. To facilitate reading and to maintain the criteria, the same type of comparison was made, as displayed in Figures 4.3 and 4.4, following the protocol described in Chapter 3.4.

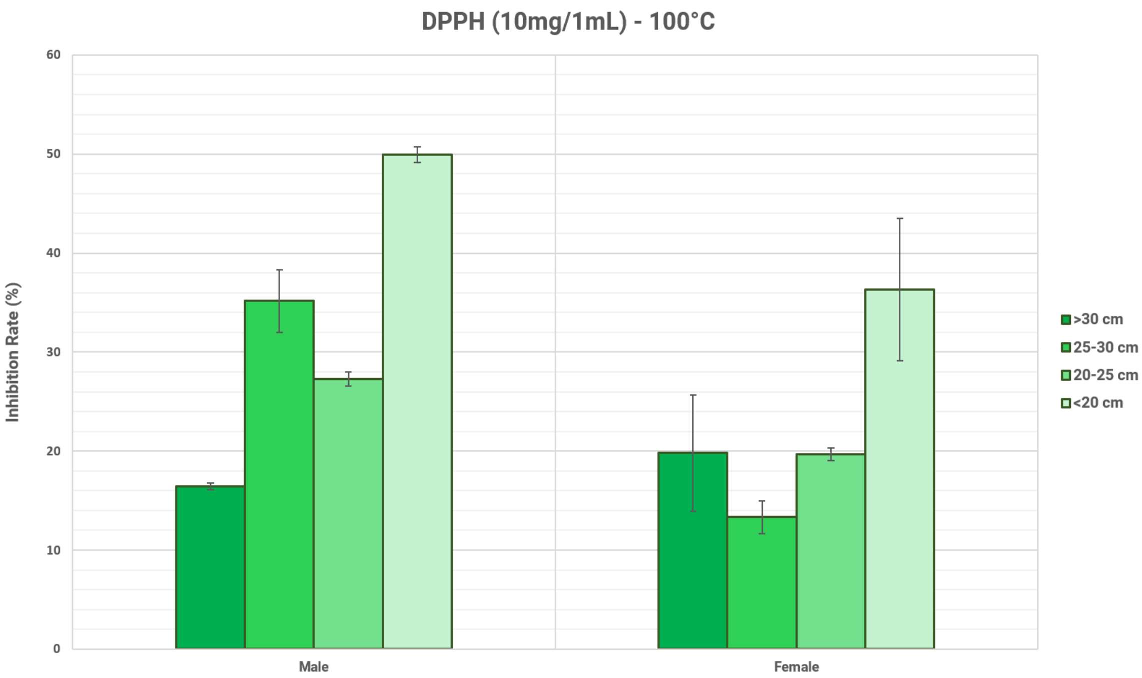

Figure 4.3. - Antioxidant activity represented by the percentage of inhibition of DPPH radical scavenging in a *F. vesiculosus* extract, obtained performing a decoction at 100°C. Data shown was obtained using a 0.1 mg/mL concentration of the extract of males and females' samples, across the four growth stages represented with respective standard deviation.

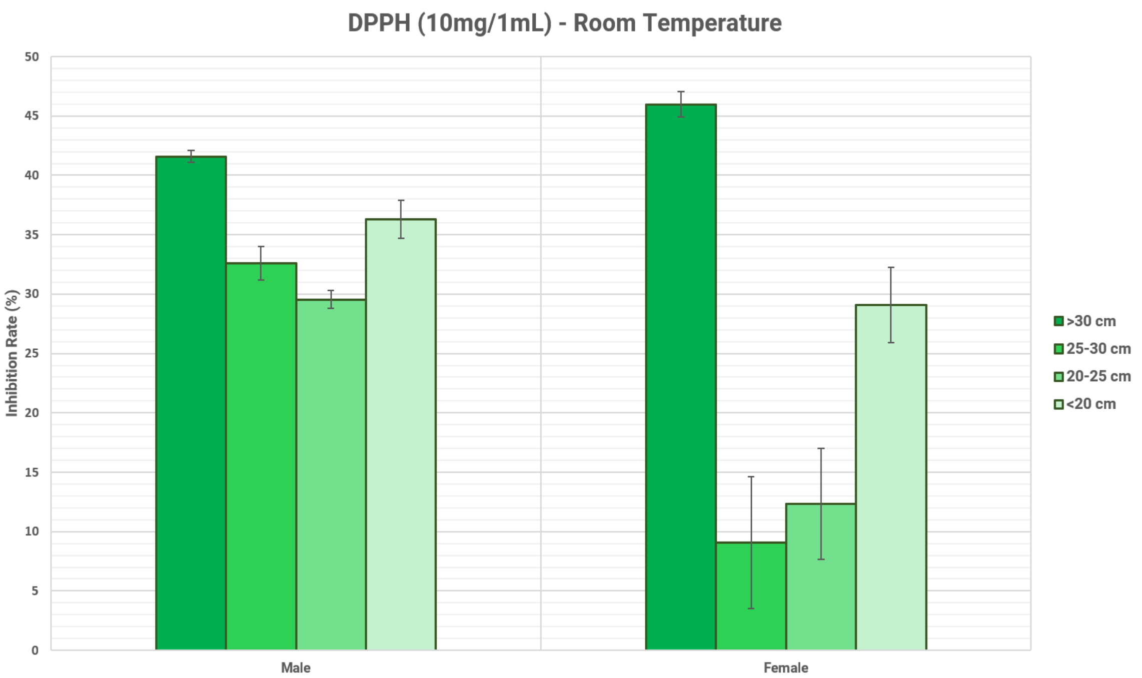

Figure 4.4. - Antioxidant activity represented by the percentage of inhibition of DPPH radical scavenging in a *F. vesiculosus* extract, obtained by aqueous extraction at 25 °C during 24 h. Data shown was obtained using a 0.1 mg/mL concentration of the extract of males and females' samples, across the four growth stages represented with respective standard deviation

Regarding the results obtained for the samples extracted at 100 ºC (Fig. 3.3), the antioxidant activity of *F. vesiculosus* is significantly affected depending on the growth stage and gender of the harvested sample. In a similar pattern to the data that was obtained about TPC, a similar pattern is observed concerning antioxidant properties of this alga. The youngest male has a significant higher inhibition rate of DPPH radical scavenging, when compared with all the other male counterparts. Interestingly, for these samples, the inhibition rate experienced a decrease when reaching the most adult stages, something that did not happen for the TPC. In a similar note, the youngest females (<20 cm) also registered the highest inhibition rate for its gender.

As the thallus is growing, phlorotannins may polymerize to more complex chains, whilst when the thallus is younger they can remain in shorter oligomeric forms [177]. These lower weight phlorotannins may well be associated with a higher antioxidant activity in *F. vesiculosus*, as Hermund et al. (2018)[179], exhibit in their work, by proposing and demonstrating a tendency to decrease in antioxidant activity as the polymerization of phlorotannin increases [179,155]. As such, and as our results show, a link can be established between these lower molecular weight phlorotannins and a higher antioxidant activity, also corroborating these results.

4.3. High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD)

In the third phase of this work, as previously mentioned, we wanted to study the differences in the components found in *F. vesiculosus* throughout its development and gender. As such, both type of decoctions were prepared and analyzed by reverse phase high-performance liquid chromatography with diode array detector (HPLC-DAD). First, we started by comparing the chromatographic profile of each growth stage within a gender and type of extract, as displayed in Figures 4.5 and 4.6.

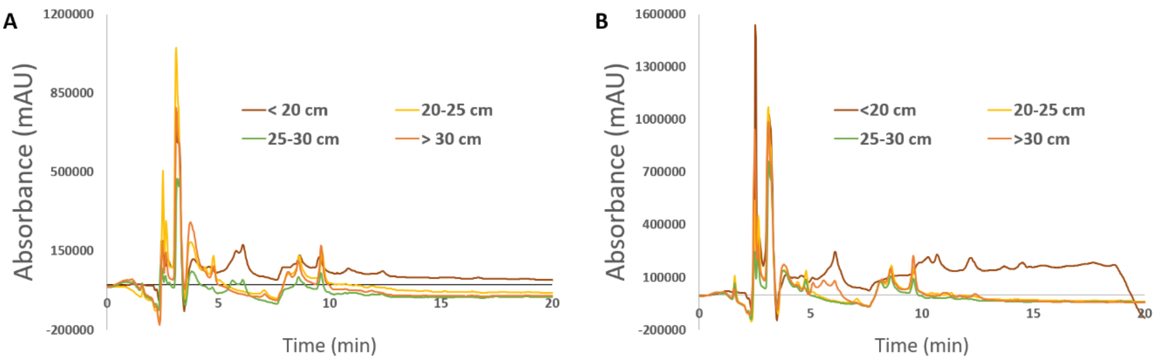

Figure 4.5 - HPLC-DAD chromatographic profile of (A) male and (B) female samples of *F. vesiculosus*, extracted at 100º C (1 mg/mL), across four growth stages - (-) <20 cm, (-) 20-25 cm, (-) 25-30 cm and (-)>30 cm. The y-axis was adjusted according to each respective maximum peaks (1200000 mAU for A and 1600000 mAU for B)

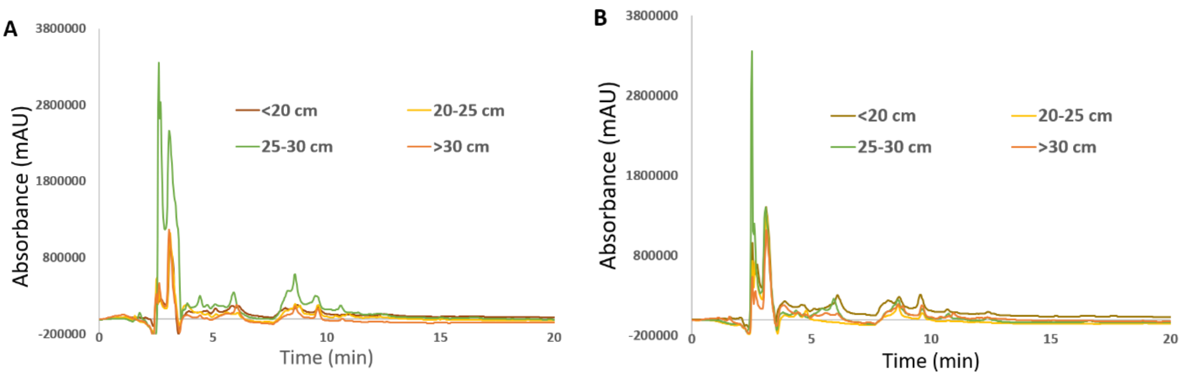

Figure 4.6 - HPLC-DAD chromatographic profile of (A) male and (B) female samples of *F. vesiculosus*, extracted at 25º C (1 mg/mL), across four growth stages - (-) <20 cm, (-) 20-25 cm, (-) 25-30 cm and (-)>30 cm. The y-axis was adjusted according to each respective maximum peaks (3800000 for both chromatograph)

The max peaks in all analysis were detected around the 3 min of retention time, however they exhibit clear differences between them. For instance, in the samples extracted at 100º C, there is one major peak at the 2,50 min mark, with a peak intensity of 1 631 062 mAU detected in the females corresponding to the <20 cm growth stage – when compared to the male counterpart, the peak intensity for the same retention time is 232 675 mAU (Table 4.1.). In addition, for the second type of extracts analyzed (25º C) there are two major peaks observed in both female and males with thalli length of 25-30 cm. In particular, in the male specimens, a peak at 2,61 min retention time with a peak intensity 3 588 842 mAU is detected, whereas at the same retention time in extracts at 100 ºC the peak intensity is only 12 858 mAU. Furthermore, another peak at 3,06 min with a peak intensity of 1 298 068 mAU is detected, while the males from extracts at 100º C only register a peak intensity of 55 220 mAU. In similar fashion, and as described, the female samples extracted at 25º C follow a similar pattern. Two major peaks are detected - at 2,50 min retention time with a peak intensity of 3 512 568 mAU and another at 3,06 min retention time with a peak intensity of 1 073 991 mAU. However, for the female samples extracted at 100ºC these same peaks have different peak intensities - of 230 893 mAU and 573 396 mAU, respectively for the retention times described.

Table 4.1. – Variation in the major peaks detected between all growth stages of the HPLC-DAD chromatographic analysis.

	Extraction at 100 °C				
		Peak intensity mAU			
	rT (min)	**<20 cm**	**20-25 cm**	**25-30 cm**	**>30 cm**
Males	**2,50**	167 244	658 162 ↗	142 372 ↘	319 923 ↗
	3,12	633 742	974 379 ↗	384 069 ↘	763 046 ↗
Females	**2,50**	1 631 062	690 644 ↘	230 893 ↘	985 860 ↗
	3,12	865 128	978 227 ↗	722 343 ↘	903 215 ↗
	Extraction at 10 °C				
		Peak intensity mAU			
	rT (min)	**<20 cm**	**20-25 cm**	**25-30 cm**	**>30 cm**
Males	**2,50**	296 205	812 881 ↗	3 588 842 ↗	553 630 ↘
	3,12	742 996	1 065 728 ↗	1 208 918 ↗	867 278 ↘
Females	**2,50**	876 388	918 319 ↗	3 512 568 ↗	689 368 ↘
	3,12	953 801	1 065 728 ↗	916 812 ↘	988 031 ↗

These differences in peaks may be indicative that there are components important to the development of *F. vesiculosus* at an intermediate stage in its development (25-30 cm). As such, certain compounds may exist in different stages of *F. vesiculosus* in different concentrations, which may have an influence as to what role they play in the seaweed's development. As retention times between genders are quite similar, it can be theorized that the same compounds appear in both female and male specimen of *F. vesiculosus.*

In another note, and trying to correlate the HPLC results to the TPC and DPPH analysis, the chromatograms of the youngest and oldest male extracts (<20 cm and >30 cm; extraction at 25 ºC) reveal a very similar pattern, especially when compared to the remaining classes (20-25 cm and 25-30 cm). As such, this may indicate the presence of similar components in these extracts that influence their total phenol concentration and antioxidant activity and thus explaining why the youngest and oldest samples have higher values when compared to the two intermediate growth stages analyzed. However, it should be taken into consideration that this tendency was not commonplace in all the results, and although promising, it cannot be entirely validated. Consequently, further studies are needed in order to establish a correspondence between the TPC, DPPH and HPLC analysis – in which identification of the compounds through Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) could reveal the presence of common key components that may influence total phenol content and antioxidant activity. What this means is that there is, in fact, a difference in phenolic content with the alga age, although the type of phenolic compounds can be the same in both genres.

4.4. Acetylcholinesterase inhibition

Although currently there is no cure for Alzheimer's Disease (AD), various therapies have been developed to diminish the symptoms of AD, especially in its early stages. One of which regards the regulation of acetylcholine (ACh), since cholinergic dysfunctions lead to lower levels of ACh in the synaptic cleft and cognitive impairment, which are two symptoms of AD[180]. Therefore, regulating the levels of acetylcholine by reducing its rate of hydrolysis in the synapses between cholinergic neurons through use of cholinesterase inhibitors, such as acetylcholinesterase (AChE) constitutes a viable method to treat these symptoms and promote cognitive function[106,181]. Due to side effects associated with some therapies with synthetic compounds[182], biological derived substrates are well desired for the regulation of AD symptoms. Various seaweed-derived compounds have been studied *in vitro* for their cholinesterase inhibition due to being rich in compounds such as phlorotannin, flavonoids and phenolic acids [106,183]. Hence, the study of this type of activity in *F. vesiculosus* and particularly the role that gender and growth may play in the acetylcholine regulation through inhibition towards acetylcholinesterase was a topic of interest in this bladderwrack characterization. Following the protocol described in Chapter 3.6, inhibition rates of all *F. vesiculosus* samples of each gender, growth stage, and extraction method were obtained in triplicates and presented in Table 4.2.

Table 4.2. Effect on the AChE activity, % of inhibition, of female and male extracts (100º C and 25º C at 1 mg/mL) of different growth stages of F. vesiculosus (n=3). n.i.: no inhibition (inhibition <1%); n.d.: not determined

	Extraction	
Growth Stage	100º C	25º C
<20 cm Female	n.i	1.52 ± 23.6
<20 cm Male	n.i	n.d
20-25 cm Female	n.i	n.i
20-25 cm Male	42.7 ± 31.7	5.41 ± 3.56
25-30 Female	n.i	n.i
25-30 Male	3.0 ± 33.3	n.i
>30 cm Female	12.1 ± 17.1	24.06 ± 0.25
>30 cm Male	n.i	n.i

Although the use of seaweed in the inhibition of AChE in phototherapeutic treatments, such as *Hypnea musciformis* (Wulfen) [184], *Ochtodes secundiramea*(Montagne) [184], *Sargassum boveanum* [185], these initial results are not in line with what is mostly described in the literature. In exception to the 25 ºC extracts belonging to the females with thalli length > 30 cm, all other samples either did not show any inhibition towards AChE or the results were too variable to assert any firm conclusion - even though *F. vesiculosus* shows high concentrations of phlorotannin that did not transcribe in a higher inhibition towards AChE. These results should be taken with some reservations since they do not align with other studies with *Fucus* – in fact, the phlorotannin detected in this alga[186] can be too big to fit inside AChE activity site – nevertheless, previous studies have revealed that a better activity was detected after purification of the extracts through and SPE column[186]. Therefore, further analysis should be performed, in better controlled conditions – the low inactivity towards AChE from the extracts obtained may be related to these not have been purified beforehand. Additionally, the presence of fucoidans and other polysaccharides that do not have an inhibitory activity towards AChE may also factor in the results. As such, purification of the samples using solid phase extraction (SPE), may originate extracts with increased inhibitory activity. Moreover, this data indicates that extractions at 100 ºC may translate in better results when compared to extracts at 25 ºC, which should be considered in future assays.

Albeit this assay was, in the most part, inconclusive, the previous antioxidant activity assessment also reveals good results for therapeutic treatments in AD. Oxidative stress plays an important role in the development of AD, as the brain is vastly composed by oxidizable lipids in addition to its high oxygen consumption [187]. *F. vesiculosus*, as previously discussed in this work, has a high antioxidant activity derived to its rich polyphenol, mainly phlorotannin, constitution and thus might constitute a valuable addition to the mitigation of the symptoms in AD.

4.5. Elemental Analysis using TXRF

For the last step of this characterization of *F. vesiculosus*, we intended to do an elemental analysis of our samples with two goals in mind: (i) to verify the nutrients reported being both present and healthy in some manner in human consumption and (ii) the presence of harmful heavy metals that could be absorbed by *F. vesiculosus* in Tagus Estuary, as was described previously in this chapter. With this in mind, we performed an elemental analysis using TXRF, described in chapter 3.7. As such, we managed to obtain information regarding this aspect, which is presented in Figure 4.7, describing each element found and its significance in µg/g of Dry Weight.

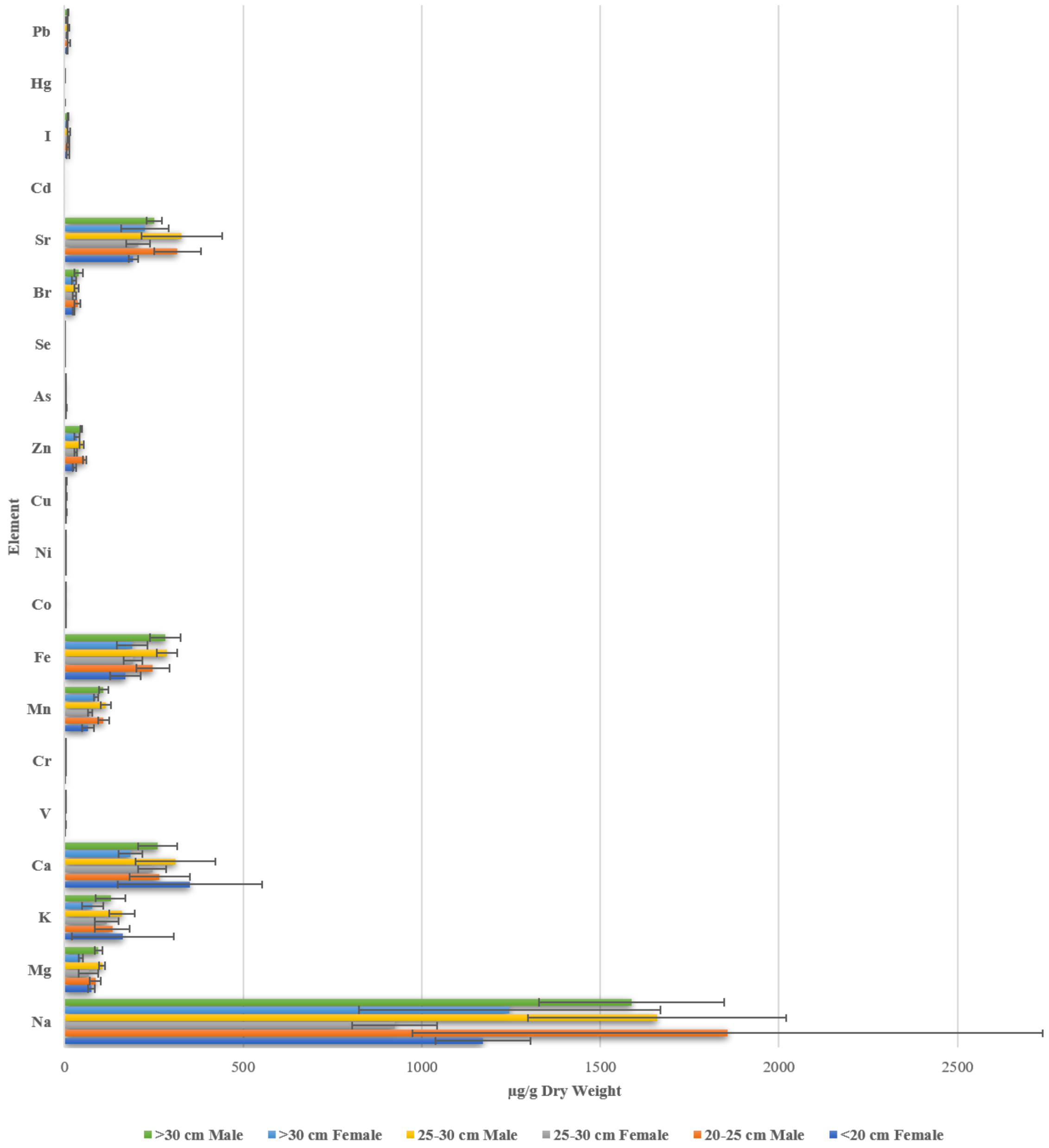

Figure 4.7. – Elements found in *F. vesiculosus*, expressed by µg/g Dry Weight, sampled from Tagus Estuary.

Below it is also presented a summary table comparing each mineral value, in Table 4.3.

Table 4.3. – Mineral Composition (mg/ 100g) of 6 different profiles of *F. vesiculosus*, comprising of 4 growth stages and both genders. (n=3)

	***F. vesiculosus* growth stage per gender analyzed**					
	<20 cm F	**20-25 cm M**	**25-30 cm F**	**25-30 cm M**	**>30 cm F**	**>30 cm M**
Na	117.12 ± 13.22	185.57 ± 88.16	92.50 ± 11.85	165.84 ± 36.20	124.59 ± 42.27	158.73 ± 25.89
Mg	7.55 ± 1.03	8.62 ± 1.51	6.72 ± 2.78	10.57 ± 0.82	4.50 ± 0.60	9.54 ± 0.98
K	16.39 ± 14.30	13.38 ± 4.89	11.77 ± 3.38	16.10 ± 3.60	7.87 ± 2.92	12.91 ± 4.24
Ca	35.08 ± 20.20	26.63 ± 8.45	24.54 ± 3.88	31.00 ± 11.15	18.49 ± 3.41	26.14 ± 5.42
V	0.06 ± 0.02	0.09 ± 0.02	0.08 ± 0.04	0.08 ± 0.01	0.05 ± 0.01	0.07 ± 0.02
Cr	0.05 ± 0.02	0.08 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.10 ± 0.01
Mn	6.49 ± 1.66	10.91 ± 1.56	7.18 ± 0.63	11.64 ± 1.40	8.83 ± 0.70	10.97 ± 1.22
Fe	17.08 ± 4.21	24.70 ± 4.59	19.12 ± 2.56	28.68 ± 2.79	18.88 ± 4.33	28.27 ± 4.30
Co	0.14 ± 0.03	0.24 ± 0.06	0.18 ± 0.02	0.28 ± 0.05	0.20 ± 0.05	0.28 ± 0.05
Ni	0.08 ± 0.02	0.14 ± 0.02	0.09 ± 0.01	0.14 ± 0.02	0.11 ± 0.02	0.15 ± 0.01
Cu	0.26 ± 0.06	0.39 ± 0.07	0.23 ± 0.02	0.39 ± 0.07	0.30 ± 0.06	0.35 ± 0.03
Zn	2.79 ± 0.57	5.57 ± 0.47	3.18 ± 0.35	4.88 ± 0.60	3.44 ± 0.66	4.59 ± 0.27
As	0.21 ± 0.13	0.37 ± 0.19	0.23 ± 0.05	0.34 ± 0.07	0.29 ± 0.07	0.22 ± 0.02
Se	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Br	2.55 ± 0.23	3.65 ± 0.85	2.71 ± 0.052	3.35 ± 0.52	2.69 ± 0.54	3.92 ± 1.19
Sr	19.22 ± 1.32	31.66 ± 6.60	20.60 ± 3.42	32.79 ± 11.28	22.47 ± 6.66	25.07 ± 2.15
Cd	0	0	0	0	0	0
I	0.99 ± 0.36	1.05 ± 0.36	1.13 ± 0.33	1.22 ± 0.38	0.74 ± 0.20	1.06 ± 0.10
Hg	0	0	0	0	0	0
Pb	0.74 ± 0.05	1.20 ± 0.40	0.72 ± 0.11	1.02 ± 0.22	0.71 ± 0.22	0.97 ± 0.15

As observed both in Figure 4.7 and Table 4.3, the main minerals found in *F. vesiculosus* are Na, Fe, K, Ca and Mg, aligning with different previous studies of this species.[153,101] Although the main mineral constituents of *F. vesiculosus* remain the same throughout different studies, their concentrations vary greatly. This may be mainly explained by a multitude of different factors involving the growth conditions of each different individual, mainly attributed to the geographical location, mineral composition of the site, pH level, salinity and water temperature [102]. Besides this, endogenous factors, such as cell wall and polysaccharide constitution may also affect the absorbance of these minerals[149].

Between each class, there were no significant differences between the concentrations of each mineral, except for the Na values, which may be attributed to the aforementioned reasons. However, being that no significant differences were found between each growth stage and gender, it is possible that such variance in Na values may be due to exogenous causes.

4.5.1. Nutritional important minerals in *F. vesiculosus*

Besides comparing values between the classes defined in our study, it is also relevant to find the meaning in those values and what benefits they bring to human health. Therefore, a comparison between various established sources for the recommended daily intake (RDI) of the minerals described in our study of *F. vesiculosus* was made and presented in Table 4.4. The values of each mineral in *F. vesiculosus* presented are considered in a supposed intake of 100 mg in the consumption of the adult m male stages of this seaweed, which showed the best results.

Table 4.4. – Comparison between various sources for the recommended daily intake (RDI) for essential minerals with the minerals detected in *F. vesiculosus*. .: FDA: Food and Drug Administration; EFSA: European Food Safety Authority; M: Male; W: Women; n.a.: not available

	Mineral recommended daily intake (RDI) vs *F. vesiculosus* mineral composition			
	FDA[188]	**National Academy**[189, 190]	**EFSA** [191]	**>30 cm M**
Na	2400 mg	2300 mg	2000 mg	158.73 mg
Mg	400 mg	M: 420 mg; W: 380 mg	M: 350 mg; W: 300 mg	9.54 mg
K	3500 mg	4700 mg	3500 mg	12.91 mg
Ca	1000 mg	1000 mg	1000 mg	26.14 mg
Cr	120 µg	M: 35 µg; W: 25 µg	n.a	100 µg
Mn	2 mg	M: 2.3 mg; W: 1.8 mg	3 mg	10.97 mg
Fe	18 mg	M: 8 mg; W: 18 mg	M: 11 µg; W: 16 µg	28.27 mg
Cu	2 mg	900 µg	M: 1.6 mg; W: 1.5 mg	35 µg
Zn	15 mg	M: 11 mg; W: 8 mg	M:16.3 mg; W:12.7 mg	4.59 mg
Se	70 µg	55 µg	70 µg	10 µg
I	150 µg	150 µg	150 µg	1.06 mg

As is evidenced by TXRF analysis, bladderwrack remains a good natural source for hypothyroidism treatment, due to its high Iodine content [192]. Iodine is particularly important for the synthesis of thyroid hormone and so the deficiency of this element may cause anomalies in the functioning of the thyroid [193]. Furthermore, iodine may enhance the functioning of superoxide dismutase, which in turn is potentially responsible for inducing apoptosis of various breast cancer cell lines, as previously studied using *L. japonica* aqueous extracts, another brown seaweed [194,195]. Moreover, iodine deficiency may have adverse effects in cardiovascular diseases [196] and thus, represents an increasingly important mineral in human diets. However, treatment of iodine deficiency using existing *F. vesiculosus* and other brown algae supplements should always be supervised by a physician, as excess consumption may also lead to thyroid disfunction, especially in individuals with pre-existing thyroid disorders. In Asian cultures, consumption of seaweed is largely common and incorporated in the respective diets. This results in a higher iodine consumptions than average, and the positive effects coming from the presence of this mineral, as long as it is not in excessive quantities, were reported [192,197].

Data also suggests that *F. vesiculosus* may constitute a good alternative source for chromium intake. Chromium, particularly its trivalent form (Cr^{3+}), which is the one humans take for its dietary benefits and is the one found in most foods [198], has its main prevalence in the treatment of type-2 diabetes as it maybe help improve blood sugar control, enhance the activity of insulin and thus help reduce cardiovascular diseases [199,200,201]. It should be noted that chromium benefits are particularly observed in individuals with a deficiency in this mineral, such as patients suffering from diabetes, and athletes, who may show low levels of chromium as exercise may lead to acute urinary chromium losses [202,203]. Therefore, supplementation of chromium should be carefully administrated.

Although manganese is widespread throughout human diet and reports in its deficiency are generally low, the lack of this nutrient may be lead to skeletal problems, as it intervenes in bone growth as well as carbohydrate, lipid and amino acid metabolism [204]. As observed, *F. vesiculosus* also constitutes a good source for manganese, although nearing the amounts for its maximum adequate intake (~11 mg/day) .

Lastly, and as was previously observed, bladderwrack is also a great source for Iron, one of the most important elements in nutrition, as its involved in many different metabolic processes. When an individual has a significant deficiency in iron, hemoglobin synthesis in erythrocyte precursors become impaired, which leads to anemia [205]. Iron insufficiency is one of the most common causes for anemia, being reported that about 50% of anemia cases are caused by an iron deficiency [206]. Iron is also important in the early stages of brain development during pregnancy, and low pregnancy iron, particularly during the 3rd trimester, is associated with adverse offspring neurodevelopment [207]. The groups most susceptible to iron deficiency are the ones who have higher demand for iron, particularly children and adolescents, due to rapid growth, women of reproductible age, due to the blood loss associated with menstruation and pregnant women, for the aforementioned reasons[207, 208]. As such, *F. vesiculosus* constitutes a valuable potential natural source for iron.

4.5.2. Heavy metals presence in *F. vesiculosus*

As previously discussed, many species of seaweed live in habitats that were exposed to pollution, and therefore may absorb harmful components, such as toxic heavy metals, whose concern regarding their impact in human health is growing [209]. Given that Tagus Estuary, from where the samples for this study were harvested from has a history of high pollution, one of the main interests of this TXRF analysis was to detect the presence of heavy metals that may have been absorbed by *F. vesiculosus* and therefore may be present in harmful concentrations. The results are expressed in Table 4.5., where there are presented values for tolerable and maximum recommended intakes of heavy metals detected in 100 mg of *F. vesiculosus*.

Table 4.5. – Heavy metals detected in *F. vesiculosus* compared against tolerable intakes defined by EFSA and WHO. b.w.:body weight; PTWI: Provisional tolerable weekly intake; UL: Upper Intake Level

	Heavy metals tolerable daily intake (TDI) vs *F. vesiculosus* mineral composition	
		>30 cm M
Ni	2.8 µg/kg b.w [210]	150 µg
As	2.1 µg/kg b.w [211]	220 µg
Pb	25 µg/kg b.w [212] TDWI	970 µg
Fe	45 mg/day [213] UL	28,27 mg
Zn	40 mg/day [213] UL	4,59 mg
Cu	10 mg/day [213] UL	35 µg

Although Tagus Estuary is a site historically affected by pollution, through receiving effluents from agricultural, industrial and urban sources and thus becoming a sink for heavy metal accumulation [162], recent studies show a stagnation on this accumulation as the treatment of effluents steadily increases [161]. Heavy metal distribution and partitioning is now mainly affected by changes in the water chemistry and bottom sediments, as an effect of the discharge areas of Lisbon drainage basins [214].

As a result of heavy metal accumulation in estuaries and other marine environments, many types of seaweed are being studied as potential species for biomonitoring these areas and particularly for their capacity to bioaccumulate heavy metals [215,216]. This type of experiment is usually done together with an analysis to the heavy metals contents in the sediments [162,217] which the vegetation or seaweed are adhered to. Although these results suggest an absorption of certain heavy metals by *F. vesiculosus*, for a proper evaluation of this seaweed biomonitoring capacity in the Tagus Estuary, a more in-depth analysis of the metals content in the sediments is needed. This data also suggests that even though there are heavy metals present in the samples harvested, their concentration do not reach nor surpass their respective upper intake levels for human consumption. Furthermore, this may be possible due to the role of physodes within the thallus. These structures are commonly found in surface cells, and have been described to potentially function as a first filter to stop heavy metals from gaining access to inner parts of the thallus[218]. At the same time, the algal cells simultaneously release the complexes of physodes that retain the incoming heavy metals thus cleansing the thallus from harmful toxins.

5. Conclusions and Future Perspectives

F. vesiculosus from the Tagus estuary, similarly to other populations of the species found in different habitats, has a high value for its phenolic content, one of its most important aspects in human health. The tendency shown for the smaller male fronds, the youngest stage of growth studied, to have a higher total phenolic content is particularly interesting, first because it was a feature shared between both types of extract (100º C and 25º C), and secondly because it correlates with their respective antioxidant activity. Furthermore, this positive aspect is reinforced by the fact that, although the more developed stages of *F. vesiculosus* had similarly higher phenolic content, it was not a direct implication of higher antioxidant activity, as samples from extractions at 25º C showed similarly high antioxidant properties. One of the main takeaways in this aspect is that, in fact, the lower molecular weight phlorotannins that are more common in younger *F. vesiculosus* individuals may contribute to the higher antioxidant potential shown. Therefore, in regard to its antioxidant potential and for its raw value in TPC, it is suggested the production and harvesting of the described younger male stages (<20 cm thallus length). Although the harvesting is simple enough to optimize its usage for their antioxidant value, since male and females are very similar in this regard, the phlorotannin content varies, and thus differentiation between male and female specimens requires laboratory work through microscopic observation which is a step that may hinder their industrial potential. Even still, in this regard, both the first two main objectives were successfully conducted.

Another interesting aspect of this characterization lies in the differences between the components present in *F. vesiculosus* found in both genders. The data obtained, albeit in different extracts, suggests variations in the components detected during the various growth stages. Thus, there may exist important components during the development of *F. vesiculosus* that play a certain role in a specific phase and therefore are found in higher concentrations. As such, if these components have an inherent value for human intake, it may be interesting exploring further, in which case the next step lies in the identification of these components.

On another note, three samples extracted at 100 ºC (20-25 cm males, 25-30 cm males and >30 cm females) show some inhibition towards acetylcholinesterase. Although only three samples out of the sixteen show some activity, this should not necessarily indicate negative value in this regard, as a more controlled assay is needed to clearly demonstrate their potential, as the results obtained offer too big of a deviation to firmly conclude *F. vesiculosus* cannot function as an acetylcholinesterase inhibitor.

Lastly, elemental analysis using TXRF further confirms previous studies on the nutritional value of *F. vesiculosus* as a good alternative source of macronutrients as Na, Fe, K, Ca and Mg as well as other micronutrients, specifically iodine, for its value in hypothyroidism treatment. On the other hand, heavy metals present in *F. vesiculosus* do not surpass their upper limits for human intake. However, further studies on this note are yet needed, particularly for the adsorption of cadmium of the individuals present in the Tagus estuary, as this was not carried out in the present work. As evidenced by Brinza et al.[158], *F. vesiculosus* can be a good bioaccumulator for cadmium, a toxic heavy metal, and therefore a further characterization of *F. vesiculosus* from Tagus estuary is needed before it can potentially be utilized for human consumption.

In summary, the present work was able to contribute new knowledge about the benefits of *F. vesiculosus* human consumption while offering new and interesting results for the optimization of its harvesting. At the same time, it opens other routes for investigation, in particular the identification of components that may offer a new *F. vesiculosus* beneficial components associated with a specific time in its development.

For future studies, one focus should aim at a better assessment in the inactivity towards AChE assay. Purification of the extracts using solid phase extraction should be considered, as it can rid the extracts of fucoidans and other polysaccharides that do not have any inhibitory activity and therefore increase the activity of the extracts. Moreover, identification of the compounds detected by the HPLC-DAD assay should be carried out utilizing Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) to better understand the results obtained from the HPLC-DAD assay and study the variation observed throughout the different stages of growth and gender of *F. vesiculosus*.

6. References

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